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EXPERIMENTAL MODIFICATION OF THE SCALE OF ORGANIZATION IN THE RECONSTITUTION OF TUBULARIA

(Eighteen figures)

C. M. CHILD
U.S. Fisheries Laboratory, Beaufort, N.C., and Zoölogical Laboratory, Duke University

THE data presented below were obtained during January, 1930, at the Laboratory of the United States Bureau of Fisheries at Beaufort, North Carolina. It is a pleasure to acknowledge my indebtedness to the Bureau of Fisheries and to Dr. Samuel Hildebrand, director of the Beaufort Laboratory, for the grant of a table and for placing at my disposal the facilities of the Laboratory.

THE HYDRANTH PRIMORDIUM OF *Tubularia*

It is a well-known fact that in the reconstitution of pieces of the *Tubularia* stem the hydranth primordium does not arise by the outgrowth of new embryonic tissue from the cut end, but by reorganization of the stem coenosarc over a certain distance proximal or distal to the cut end, according as the hydranth arises at the distal or the proximal end of the piece. The tentacles arise as longitudinal coenosarcal ridges in this region and separate from the body of the hydranth by constriction progressing longitudinally from the tip of each tentacle ridge basipetally. Figure 1 represents a characteristic primordium at a stage when the tentacle ridges have become clearly visible. In later stages the region of the stem below the hydranth elongates and this elongation first compresses the developing hydranth longitudinally and finally pushes it out of the perisarc.

In a stem of a certain diameter the length of the primordium is the chief factor in determining the size of the resulting hydranth at the time of emergence. In other words, the size of the hydranth is not determined by the growth of its various parts to a certain size but by the localization of these parts at certain distances from the level of section in the coenosarc of the stem as substratum. Under given conditions these localizations take place on a certain scale so that it is possible to speak of the scale of organization of the hydranth primordium. In view of these facts it is of interest to raise the question whether and how the scale of organization can be altered and controlled experimentally. If such alteration and control are possible they represent alteration and control of localization of parts and of the distance between the levels of the stem at which particular parts are determined. This localization and determination take place in a general cellular substratum the various levels of which are not in any way predetermined for the development of particular parts of a hydranth. The level of section of the stem represents the primary factor in determining the localization and reorganization which follows. If we can alter and control the distance from the level of section at which these various localizations take place, the only possible conclusion seems to be that in so doing we alter the range of a localizing factor or factors originating primarily at the level of section and determining the scale of organization of the primordium. The present paper is chiefly concerned with data which show that such control is possible.

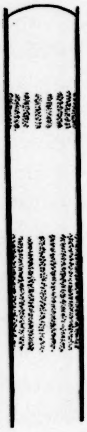


FIG. 1

Earlier work of Driesch (1899), Peebles (1900, 1902), Morgan (1903), and Child (1907*a*, *b*) has shown that in the reconstitution of pieces of the *Tubularia* stem the length of the hydranth primordium and the size of the resulting hydranth may differ greatly in relation to various physiological factors. In general, the lengths of both distal and proximal primordia decrease as the distance from the apical end of the stem of the level at which they develop increases, but level is not the only factor concerned, for a distal primordium is in general longer than a proximal at the same level. In pieces only a few millimeters in length, particularly those from the distal stem regions, the scale of organization of the primordium may differ greatly in different cases. In some such pieces the whole length of the piece may be included in unipolar or bipolar hydranth primordia or in primordia of only the distal portions of hydranths, e.g., manubria without proximal tentacles. Other pieces of the same length from the same level of stem may give rise at one or both ends to hydranth primordia so short that more or less of the coenosarc of the piece remains as stem. These differences apparently depend on difference in physiological condition in different stems. It has been shown by Driesch (1899) and Child (1907*b*) that length of piece is a factor in determining length of primordium. In pieces below a certain length the length of the primordium decreases in general with further decrease in length of piece, but the decrease in length of primordium is in general not proportional to the decrease in length of piece, so that in pieces below a certain length the primordium may be as long as the piece or even longer. In the latter case only the distal part of the primordium develops. In such short pieces the partial primordia are often on a larger scale of organization than the primordia in somewhat longer pieces.

These facts make it evident that the scale of organization of the primordium may differ widely even in the same stem with difference in level of section, in position at the distal or proximal end of the piece, in length of piece, and in physiological condition of individual stems. These facts alone, however, do not throw much light on the question of the nature of the factors concerned in determining the differences.

If a physiological gradient exists in the *Tubularia* stem the fact that primordium length decreases in general as the level of section becomes more proximal, at least to a certain limit in long stems, suggests that quantitative metabolic differences may be factors in determining primordium length. Also the fact that individuals which appear to be in poor condition usually develop shorter primordia than those in good condition appears to point in the same direction.

The decrease in length of primordium in pieces below a certain length suggests that the proximity of the changes going on at the other cut end may in some way decrease the scale of organization. We know that development of a hydranth at one end of a piece retards hydranth development at the other end. Since a cut end is the primary factor in determining hydranth development in pieces, it seems probable that the presence of a cut end, or more specifically of the activities resulting from section, may decrease the scale of organization at the other end if sufficiently near it.

These various facts and the conclusions which they suggest have led to the attempt to alter the scale of organization of the primordium by means of external agents. A few experiments along this line which were performed some years ago have been briefly mentioned elsewhere (Child, 1915). The results of further experiments, chiefly with KCN as agent, are recorded below.

MATERIAL AND METHOD

The *Tubularia* material was abundant and in good condition at Beaufort during January, 1930. Stocks were collected at frequent intervals and kept in running sea water and pieces were usually cut and subjected to experimental conditions within 24 hours after collection, or the day following collection, and in no case was material used which had been more than 3 days in the laboratory, though the hydranths were apparently still in good condition at the end of this period. The experiments consisted simply in cutting and subjecting to experimental conditions pieces of various lengths from the distal 10–20 mm. of stems 5–10 cm. in length which bore large hydranths apparently in good condition. Stems of approximately equal length from a single collection and, with very few exceptions, from the same group of individuals were used in each series. Pieces were cut in lots of fifty and in various lengths as follows: 10–12 mm., one piece from a stem; 6–7 mm., two pieces in succession from each stem; 4–5 mm., five pieces in succession from each stem; 2–3 mm., five pieces from a stem. Lots of fifty pieces were subjected to solutions of KCN ranging from m/50,000 to m/150,000 in concentration and for various lengths of time from 48 hours to the whole period of reconstitution. A few lots were subjected to sea water brought to pH 7 by addition of HCl with a minimum of agitation in order to avoid removal of CO_2. In all cases about 400 cc. of solution in a closed container with only a small bubble of air were used for each lot, and with the longer periods of exposure the solution was renewed every 48 hours, or in some experiments more frequently. Each experimental lot was accompanied by a sea-water control of fifty pieces prepared at the same time from the same group of individuals, except in a few cases in which one sea-water control was run with more than one experimental lot, all being taken from the same stock at the same time.

Except for rare failures to undergo reconstitution, all the longer pieces developed complete hydranth primordia and the lengths of as many as possible of the primordia at the distal ends of the pieces were measured as they reached the proper stage. Measurements were made by means of an ocular micrometer, 16 divisions equaling 1 mm., the distance from the distal end of the coenosarc to the proximal end of the proximal tentacle ridges being taken as the primordium length. In the shorter pieces measurements were made only of complete primordia. Many of these pieces, particularly those 2–3 mm. in length, gave rise to unipolar or bipolar partial primordia, consisting of more or less of the distal hydranth region only. Although these were not measured, a record was made in each case of the portion of the hydranth or hydranths developed and of the unipolarity or bipolarity. The numbers of primordia measured in different lots differ widely for two reasons: first, in some cases the stages during which measurement could be made were passed during the night and on the following morning the hydranth had already emerged or was compressed longitudinally by elongation of the region proximal to it so that its original length as primordium could not be determined; second, the number of partial primordia which were not measured differs in different data.

EXPERIMENTAL DECREASE IN LENGTH OF PRIMORDIUM

Data for the longer pieces are given in Table I; the column headings render detailed explanation of the table unnecessary. It will be observed from the third column that various concentrations of KCN were used and that the pieces remained in the higher concentrations, m/50,000, m/75,000, m/100,000, only 48 or 72 hours, after which they were washed and returned to sea water. In these concentrations primordia did not develop during the period of exposure, except rarely in m/100,000 and even after return to sea water development was retarded. In m/150,000, however, primordia developed in the solution, though more slowly than in sea water. The last two lots of Table I, VII and VIII of Series 3, give data for sea water brought to pH 7 by addition of HCl, Lot VII with 72 hours' exposure, Lot VIII with continuous exposure. The fourth column of the table shows that the number of primordia measured differs widely in different lots. In Series 3 I and II two control lots of 50 pieces each are combined and 67 primordia were measured in the

TABLE I

EFFECT OF KCN AND OF SEA WATER AT PH 7 ON LENGTH OF HYDRANTH PRIMORDIA IN PIECES 10–12, 6–7, AND 4–5 MM. IN LENGTH

Each lot consists of 50 pieces: 10–12-mm. pieces, 1 from distal region of each stem; 6–7-mm. pieces, 2 in succession from distal region of each stem; 4–5-mm. pieces, 5 in succession from distal region of each stem. Primordium lengths are given in micrometer divisions.

Series and Lots		Lengths of Pieces in Mm.	Experimental Conditions	Numbers of Primordia Measured	Average Lengths of Primordia	Percentages of Decrease in Length
1	I	10–12	Sea-water control	49	60.2	....
	II		KCN m/150,000; continuous	37	54.5	9.4
2	I	6–7	Sea-water control	20	46.7	....
	II		KCN m/100,000; 72 hours	38	36.7	21.4
	III		KCN m/150,000; continuous	30	36	22.9
3	I II	4–5	Sea-water control	67	35.25	....
	III		KCN m/50,000; 48 hours	36	27.4	22.2
	IV		KCN m/75,000; 72 hours	9	29.2	17.2
	V		KCN m/100,000; 72 hours	9	33.3	5.5
	VI		KCN m/150,000; continuous	41	31.3	11.2
	VII		Sea water; pH 7, 72 hours	35	29.1	17.4
	VIII		Sea water; pH 7, continuous	26	23.2	34.2

two lots. All others are lots of 50 pieces each. In Series 3 IV and V the death-rate was high and only 9 primordia were measured in each case. In all other lots 20 or more were measured.

In the fifth column of the table the average primordium lengths are given for each lot in divisions of the ocular micrometer, 16 divisions to the millimeter. The length, 35.25, of the controls in Series 3 I and II represents the average length for two control lots, totaling 100 pieces. The average lengths for the two lots were respectively 36.8 and 34.

From the fifth and sixth columns it will be noted that the decrease in primordium length in 10–12-mm. pieces (Series 1 I and II) resulting from continuous exposure to KCN m/150,000 is 9.4 per cent. In 6–7-mm. pieces (Series 2 I, II, and III) it is 21.4 per cent after 72 hours' exposure to KCN m/100,000 and 22.9 per cent with continuous exposure to KCN m/150,000. In Series 3 III it is 22.2 per cent after 48 hours in KCN m/50,000; in Series 3 IV, 17.2 per cent after 72 hours in KCN m/75,000; and in Series 3 V, only 5.5 per cent after 72 hours in KCN m/100,000. The slight decrease in length in this lot results from the fact that the primordia measured were those which showed the greatest degree of recovery from KCN. Most of the other pieces failed to recover sufficiently to develop primordia and died within a few days after return to sea water. In all lots with temporary exposure to KCN the pieces which show the greatest degree of recovery and develop primordia earliest after return to sea water show, as might be expected, in general the least decrease in primordium length. If other pieces of this lot had recovered sufficiently to develop primordia the average decrease would undoubtedly have been much greater. Series 3 VI, with continuous exposure to KCN m/150,000, shows 11.2 per cent decrease in length, almost the same as Series 1 II, a lot of longer pieces under similar conditions.

Series 3 VII and VIII in sea water pH 7, VII with 72 hours' exposure, and VIII with continuous exposure show decreases of 17.4 and 34.2, respectively, that is, a decrease twice as great with continuous as with temporary exposure. This difference in the two lots is undoubtedly greater than it should be because in Lot VIII some of the earlier, longer primordia were not measured, but less decrease in length is to be expected in Lot VII than in Lot VIII because some degree of recovery undoubtedly occurred after return of Lot VII to sea water. The table shows very clearly that primordium length is decreased by the experimental conditions. Aside from the variations in results due to failure to obtain measurements of all primordia, the differences in percentages of decrease in the different lots are doubtless due in part to the difference in experimental conditions and in part to difference in susceptibility in different lots.

Measurements of primordium length in pieces 2–3 mm. in length were obtained from eight series, each consisting of two lots of 50 pieces each, one a sea-water control, the other subjected either temporarily or continuously to KCN in concentrations ranging from m/50,000 to m/150,000. The data from these eight series are given in Table II. The first four columns of the table require no explanation. In the fifth column the averages for all controls and all experimental lots of Series 1, 2, and 3 and Series 6, 7, and 8 are given, that is, the general averages for 48 hours' exposure to KCN m/50,000 and for continuous exposure to KCN m/150,000. The sixth column gives the percentages of decrease in primordium length for the various concentrations and periods of exposure, the average length of the control being taken as 100 per cent in each case.

The measurements on which the averages of Table II are based include most of the complete primordia which developed. In some of the control lots, e.g., 1 I and 6 I, almost all of the pieces developed only partial primordia, and the few complete primordia which did develop appeared very late and were short, consequently their lengths could justly be compared only with those of the experimental lots which developed latest. For this reason the few measurements made in these two controls are omitted from the table. In Series 7 also the number of partial primordia was relatively large in both control and experimental lots, consequently the numbers of complete primordia measured are small.

Table II shows a decrease in primordium length in the experimental lot in every series except Series 2, in which control and experimental lengths are practically identical. This series is the only series prepared from material which had been kept as long as 3 days in the laboratory. The primordia of the control lot developed more slowly and, as the table shows, are considerably shorter than those of other control lots. In Series 7, on the other hand, the pieces were cut within a few moments after the animals were collected, and here the length of the control primordia and the decrease in length in KCN are much greater. Apparently the 3-day period in the laboratory in the case of Series 2 altered the physiological condition of the material to such an extent that the primordium length in the con-

TABLE II

EFFECT OF KCN ON LENGTH OF HYDRANTH PRIMORDIUM IN PIECES 2–3 MM. IN LENGTH

Each series consists of one control and one experimental lot of 50 pieces each. Each lot consists of 5 pieces cut in succession from the distal region of each of 10 stems. Primordium lengths are given in micrometer divisions.

Series and Lots	Experimental Conditions	Numbers of Primordia Measured	Average Lengths of Primordia	General Averages for Different Concentrations of KCN	Percentages of Decrease in Length
1 I........	Sea-water control	....	......	Controls 25.4; m/50,000 22.2	12.6
1 II.......	KCN m/50,000; 48 hours	20	20.7		
2 I........	Sea-water control	25	23.7		
2 II.......	KCN m/50,000; 48 hours	14	23.8		
3 I........	Sea-water control	20	27.5		
3 II.......	KCN m/50,000; 48 hours	32	22.5		
4 I........	Sea-water control	27	28.2	....	19
4 II.......	KCN m/100,000; 48 hours	39	23		
5 I........	Sea-water control	27	29.5	....	13.4
5 II.......	KCN m/125,000; continuous	25	25.4		
6 I........	Sea-water control	....	......	Controls 33.7; m/150,000 25.3	24.6
6 II.......	KCN m/150,000; continuous	19	31		
7 I........	Sea-water control	10	41.2		
7 II.......	KCN m/150,000; continuous	9	26.3		
8 I........	Sea-water control	11	26.8		
8 II.......	KCN m/150,000; continuous	24	20.4		

trols was considerably decreased and the susceptibility to KCN was also decreased to such an extent that no further decrease in length was brought about by the experimental conditions. According to the last column of the table the decrease in length after KCN m/50,000 for 48 hours is only about half as great as with continuous exposure to KCN m/150,000. In the higher concentration there is no indication of primordium development until after return to water. Hydranth primordia begin to appear 24 hours or more after return to water, most of them only after 48 hours or more. Except in Series 2, in which, as noted above, development was slow, most of the control primordia are fully developed within 48 hours after section. Evidently KCN m/50,000 completely or almost completely inhibits hydranth development during the period of exposure. In fact, pieces which remain in this concentration usually die without reconstitution. The development of the primordium takes place only after some degree of recovery in sea water from the effect of the KCN. That such recovery is not complete, however, is indicated by the fact that development is less rapid, even after return to water, than in the controls and by the decrease in primordium length. Without such recovery development of primordia would occur only very rarely or not at all.

Undoubtedly the decrease in primordium length in Series 1–3, viz., 12.6 per cent, is considerably less than it would be if the full effect of the experimental conditions appeared, for in Series 1 I the scale of organization was so large in the controls that almost all pieces gave rise to partial primordia and were not measured. The few wholes which did appear and were measured were those which developed most slowly and possessed the shortest primordia, consequently they are omitted from the table. Because of this procedure the large scale of organization in this control does not appear at all in the primordium lengths. Moreover, as already noted, the control lengths in Series 2 are decreased by the 3-day period in the laboratory. If we assume that the average primordium length in Series 1 I is 33—as a matter of fact it is undoubtedly more than that—and that the average length in Series 2 I when the material was fresh was 28, which is about that in most other controls, the decrease in primordium length in Series 1–3 becomes 24.7 per cent. In other words, the decrease in length would be at least as great as this if the full effect of experimental conditions appeared.

Series 4 and 5 each consist of single control and experimental lots, and a considerable number of primordia were measured in each lot. In Series 4 the material was fresh, therefore it is probable that this series shows approximately the effect of the experimental conditions. The material of Series 5 was in the laboratory 2½ days before being used and the decrease in length, 13.9 per cent, is less than in fresh material.

In Series 6–8 the decrease in length, 24.6 per cent, cannot be regarded as representing exactly the effect of the particular experimental conditions, for in Series 6 I as in Series 1 I the scale of organization was so large that most pieces gave rise to partial primordia and were not measured. Since the few measurements made are not included in the table because they represent only the later shorter primordia, this large scale of organization does not appear in the data as tabulated. In several of the other lots only small numbers of primordia were measured.

Summarizing the data of Table II, all series of pieces 2–3 mm. in length, with the exception of Series 2 from material 3 days in the laboratory, show a decrease in primordium length ranging from 18.3 to 33.4 per cent for single series and from 12.6 to 24.6 per cent for different concentrations and periods of exposure to KCN. The differences in percentage of decrease under different experimental conditions are of uncertain significance because the measurements of complete primordia do not take into account the numbers of partial primordia which develop. This number differs widely in different series and, as will appear in the following section, is always greater in the control than in the experimental lot.

In addition to the data of Table II one lot of 50 pieces developed in sea water brought to 7.3 by addition of HCl with minimum agitation. The control for this lot was the control of Series 3 (3 I [Table II]). The decrease in primordium length in this experimental lot as compared with the control was 9 per cent, but since the death-rate was 24 per cent this decrease may not represent the full effect of the conditions.

In Table III the average primordium length for all the 2–3-mm.

controls taken together, viz., 28.4, is considered as the standard length, and the decreases in length under the different experimental conditions are given in percentages of this length. According to Table III the decrease in length is greatest in KCN m/50,000 with 48 hours' exposure and least in m/125,000 and m/150,000 with continuous exposure, a result more or less opposed to that of Table II. This method of treatment of the data is in certain respects more satisfactory than that of Table II since the absence of measurements in Series 1 I and Series 6 I and the shortness of the primordia in Series 2 I do not affect the results to so great an extent. On the other hand, the data as given in Table III do not show the full effect of KCN in Series 6–8 because the average primordium length for all controls, 28.4, is considerably less than the average length for these three series, which is 33.7. These differences with difference in method of treatment of the data show that the procedure of measuring only complete primordia in these short pieces, while serving to demonstrate that primordium length is decreased by the experimental conditions, is not satisfactory for the purpose of comparison of the effects of the different conditions.

TABLE III

AVERAGE PRIMORDIUM LENGTHS AND PERCENTAGES OF DECREASE IN LENGTH IN 2–3-MM. PIECES FOR DIFFERENT CONCENTRATIONS OF KCN AND DIFFERENT PERIODS OF EXPOSURE AS COMPARED WITH THE AVERAGE LENGTH FOR ALL CONTROLS

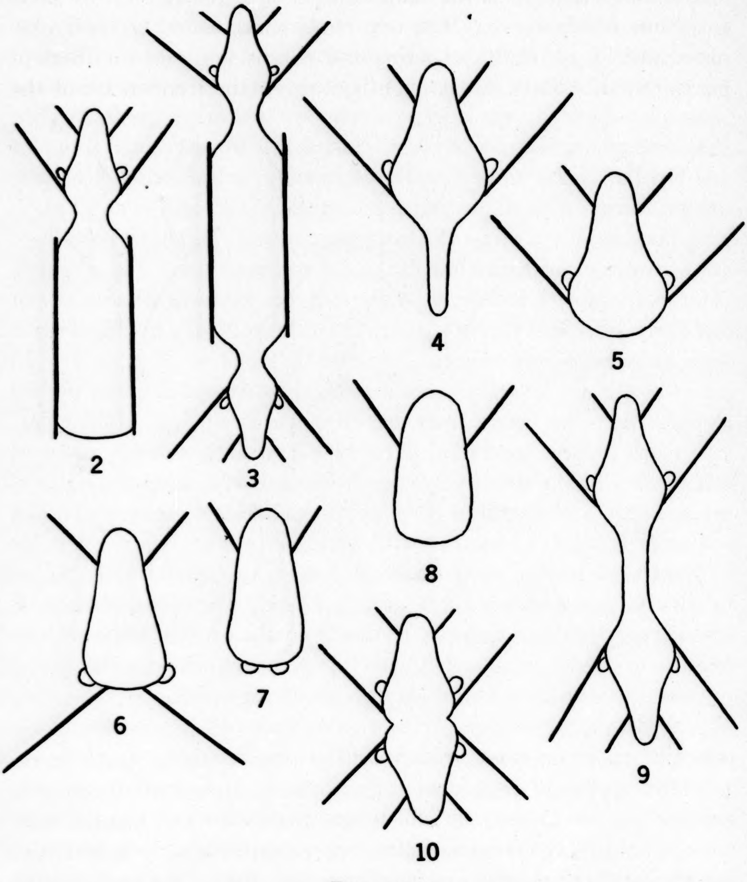
	Sea-Water Controls	KCN m/50,000 48 Hours	KCN m/100,000 48 H	KCN m/125,000 Continuous	KCN m/150,000 Continuous
Numbers of pieces.......	400	150	50	50	150
Numbers of primordia measured............	120	66	39	25	52
Average lengths.........	28.4	22.1	23	25	25.1
Percentages of decrease in length..............	....	22.9	19	12	11.6

Tables I–III show beyond question that the scale of organization of the hydranth primordium can be altered by external factors. That such alteration is possible is a fact of considerable significance for the conception of development, for it means that such external factors may play a part in localization and determination of parts as well as in growth.

EXPERIMENTAL ALTERATION IN THE FREQUENCIES OF DIFFERENT FORMS

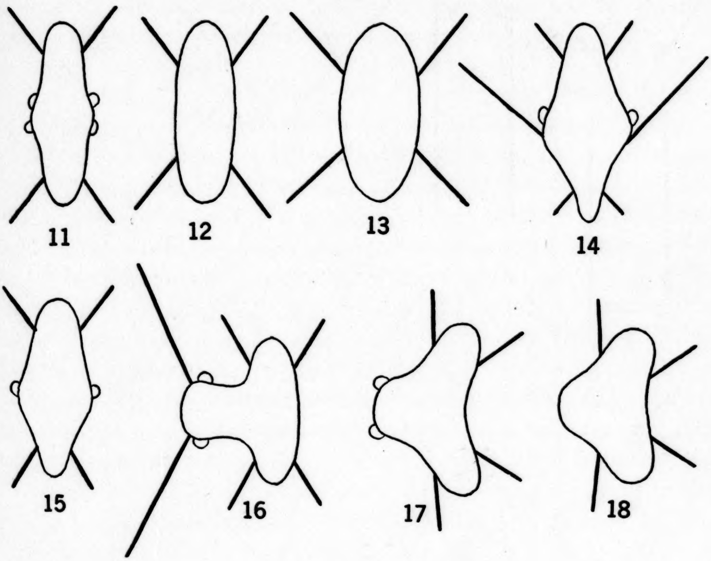
For the purpose of consideration of the frequencies of the different forms which develop from pieces three regions of the body are distinguished, the hydranth primordium and the hydranth developing from it, the stalk consisting of the short naked region below the hydranth, the stem consisting of coenosarc inclosed in perisarc. The various forms which develop are classified in four groups, unipolar and bipolar wholes and unipolar and bipolar partial forms. Unipolar wholes consist of a single distal hydranth, stalk, and stem (Fig. 2) with occasionally a stolon at the proximal end. Bipolar wholes consist of two hydranths with stalks and a common stem (Fig. 3). Unipolar partial forms include all those unipolar forms in which more or less of the proximal region is absent and range from a hydranth with stalk to a manubrium with distal tentacles only (Figs. 4–8). In pieces of a given length the scale of organization of unipolar partial forms is of course larger than that of unipolar wholes. Bipolar partial forms possess the distal parts of a hydranth at each end and other parts in the usual sequence from each end so far as scale of organization and length of piece permit. Such forms may consist of complete bipolar hydranths with common stalk (Fig. 9), bipolar hydranths without stalk (Fig. 10), bipolar manubria with distal tentacles and gonophore buds (Fig. 11), bipolar manubria with distal tentacles only (Figs. 12, 13), or development of the two polarities may be unequal (Figs. 14, 15), or finally forms with bipolar distal regions and a common unipolar proximal region may occur (Figs. 16–18). In pieces of a given length the scale of organization of bipolar partial forms is larger than that of bipolar wholes.

In this species of *Tubularia* at the time the experiments were performed the 10–12-mm. and 6–7-mm. pieces always gave rise to wholes, that is, the length of piece was always greater than that of the hydranth primordia and stalks so that some part of the piece remained as stem as in Figures 2 and 3. The 4–5-mm. pieces very rarely gave rise to partial forms. Almost all of the pieces 4–5 mm. or more in length gave rise to unipolar wholes with hydranth at the distal end (Fig. 2). A few pieces developed a stolon at the proximal end and a few gave rise to bipolar forms like Figure 3. Effects of the experimental conditions on the form frequencies in these longer pieces were either absent or so slight as to possess no significance.



FIGS. 2–10

In the pieces 2–3 mm. in length, however, unipolar and bipolar partial forms appeared much more frequently and the bipolar partial forms were more frequent in the controls. In the experimental lots the frequencies of wholes and also of unipolar forms showed a very considerable increase over the controls. The data concerning the form frequencies in 2–3-mm. pieces are given in Table IV. The material of Table IV is the same as that of Table II and the same series numbers are used. The first three columns of the table give series and lot numbers, experimental conditions, and numbers of pieces. In the fourth to the eighth columns the frequencies of the various forms as defined above and the frequencies of death or no development are given in percentages. The ninth and tenth columns give the percentages of all wholes, both unipolar and bipolar, and the percentages of increase in the experimental lots. The last two columns give the percentages of all unipolar forms, both whole and partial, and the percentages of increase in the experimental lots. Series 1–3 and 6–8 are combined in the table because in each case the three series represent the same experimental conditions.



FIGS. 11–18

The fourth and fifth columns, "Unipolar Wholes" and "Bipolar Wholes," and the ninth column, "Total Wholes," which is the sum

TABLE IV

FORM FREQUENCIES IN PERCENTAGES FROM 2–3-MM. PIECES IN SEA WATER AND DIFFERENT CONCENTRATIONS OF KCN WITH DIFFERENT PERIODS OF EXPOSURE

Series and Lots	Experimental Conditions	Numbers of Pieces	Unipolar Wholes	Bipolar Wholes	Unipolar Partial Forms	Bipolar Partial Forms	Dead or No Development	Total Wholes	Percentages of Increase of Wholes	Total Unipolar Forms	Percentages of Increase of Unipolars
1–3 I.	Sea-water control	150	46	3.3	14	30.6	6	49.3	. . . .	60	. . . .
1–3 II.	KCN m/50,000; 48 hours	150	72	10	2	10	6	82	64.3	74	23.3
4 I.	Sea-water control	50	66	8	6	16	4	74	. . . .	72	. . . .
4 II.	KCN m/100,000; 48 hours	50	74	10	6	10	0	84	13.7	80	11.1
5 I.	Sea-water control	50	30	6	8	52	4	36	. . . .	38	. . . .
5 II.	KCN m/125,000; continuous	50	54	16	4	22	4	70	94.4	58	52.6
6–8 I.	Sea-water control	150	27.3	4	12	44.7	12	31.3	. . . .	39.3	. . . .
6–8 II.	KCN m/150,000; continuous	150	42	4.7	12.7	35.3	5.3	46.7	48.9	54.7	39.4

of the fourth and fifth, show that the frequencies of both unipolar and bipolar wholes increase in the experimental lots, the increase in bipolar wholes being very slight, that of unipolar wholes much greater. The tenth column shows that the increase in frequency of all wholes ranges from 13 to 94.4 per cent. The combination of the data of Series 1–3 and 6–8 does not alter the results except as regards percentages of increase, for each series shows an increase in frequency of wholes which ranges from 12.1 per cent in Series 3 to 1,270 per cent in Series 1. The very wide range of variation in the increase of wholes results chiefly from the fact that different stocks of *Tubularia* show very different frequencies of wholes in the controls, the differences ranging from 6 to 90 per cent. These differences evidently depend on the physiological condition of the material and this in turn depends in part on the length of time the material was kept in the laboratory and apparently on conditions in nature, perhaps on variations in the salinity of the water in the tidal estuary from which the animals were collected. The important point is that whether the frequencies of wholes were high or low in the controls, they were always higher in the experimental lots from the same stock and run at the same time. In all series in which the frequencies of wholes were low in the controls the increase in the corresponding experimental lots was great, 370–1,270 per cent, while in those series in which the frequencies of wholes were high in the controls the increase in the corresponding experimental lots was much less, 12.1–31.7 per cent.

This increase in frequency of wholes in the experimental lots is very evidently associated with the decrease in length of primordium shown in Table II. With decrease in length of primordium, that is, with a smaller scale of organization, a portion of the piece more frequently remains as stem. In those series in which the frequencies of wholes are low in the controls the decrease in the primordium length under experimental conditions is sufficient to determine that a large number of pieces shall produce wholes instead of partial forms, but in the series with high frequencies of wholes in the controls the number of pieces which produce wholes instead of partial forms under the experimental conditions is of course much smaller.

The percentages of increase of wholes with different concentrations do not permit definite conclusions concerning the difference in effects of the different experimental conditions. If such differences in effect exist, they do not appear because of the wide variation in the frequencies of wholes in the different stocks. Moreover, the percentages of increase of wholes do not run parallel to the percentages of decrease in primordium length in Table II. The wide variation in different stocks and also the fact that only complete primordia were measured obscure any parallelism that might exist. The data of Table IV, however, do show beyond question that the frequencies of wholes in 2–3-mm. pieces are increased by the experimental conditions.

The eleventh column of Table IV, "Total Unipolar Forms," gives the percentages of all unipolar forms, both whole and partial, and the last column gives the percentages of increase of unipolar forms in the experimental, as compared with the control lots. This column shows that in all cases an increase occurs, the percentage ranging from 11.1 to 52.6. The percentages of increase evidently depend chiefly on the frequencies of unipolar forms in the controls rather than upon the particular experimental conditions. The lower the frequency in the controls, the greater the increase in the experimental lots and vice versa. Nevertheless, the fact remains that the frequencies of unipolar forms increase under all the experimental conditions and the percentage increase is greater in the lower concentrations with continuous exposure (Series 5, 6–8) than in the higher concentrations with continuous exposure (Series 1–3, 4).

In Table V the percentage frequencies of wholes and unipolar forms for all controls taken together and for the different experimental conditions, and the percentage increases of wholes and unipolar forms over the total control frequency, are given. According to this table the increase is greatest in the two higher concentrations and so slight as to be of little significance in the lowest concentration. Comparing Table V with Table III, it is evident that there is a general parallelism between the data on primordium length and the data on increase in frequency of wholes. In other words, the higher concentrations of KCN which give the greatest decreases in primordium length also give the greatest increases in frequency of wholes and the lowest concentration gives both the least decrease in primordium length and the least increase in frequency of wholes. A closer parallelism between the two sets of data cannot be expected since the primordium measurements include only a part of the pieces, while the form frequencies include all.

The increases in unipolar forms in Table V change in the same direction as the increases in wholes, being greatest in the two highest concentrations and least in the lowest. This is essentially the opposite of the effect as shown in Table IV. This difference is apparently due to the fact that the controls of some of the single series show a very high frequency of bipolar partial forms, and in these the experimental conditions determine a greater percentage increase of unipolar forms than in those series in which the controls show a low bipolar frequency. It so happens that the high bipolar frequencies occur chiefly in the controls for the lower concentrations. In the total frequency for all controls these differences are at least in part eliminated. This being the case, Table V probably represents more nearly than the last two columns of Table IV the effects of the different experimental conditions on polarity.

TABLE V

SUMMARY OF TABLE IV GIVING FREQUENCIES OF WHOLES AND UNIPOLAR FORMS IN PERCENTAGES AND PERCENTAGES OF INCREASE IN FREQUENCY FOR THE DIFFERENT EXPERIMENTAL CONDITIONS AS COMPARED WITH THE TOTALS FOR ALL CONTROLS

	Sea-Water Controls	KCN m/50,000 48 Hours	KCN m/100,000 48 Hours	KCN m/125,000 Continuous	KCN m/150,000 Continuous
Numbers of pieces.......	400	150	50	50	150
Wholes................	44	82	84	70	46.7
Percentages of increase of wholes..............	....	86	91	59	6
Unipolar forms..........	51	74	80	58	54.7
Percentages of increase of unipolars............	....	46	57	14	7

The increase in unipolarity under experimental conditions shows that the effect of these conditions on hydranth development is not the same at the two ends, even of pieces as short as these. The differential effect indicates the existence of a quantitative difference in physiological condition at the two ends—in other words, a gradient. With the two higher concentrations of KCN, m/50,000 and m/100,000, the primordia do not appear until a day or more after return to water, that is, until some degree of recovery from the effects of KCN has occurred. The increase in unipolarity under these conditions indicates that such recovery is differential, the distal region recovering more rapidly than the proximal. This differential is to be expected if a gradient is present since the distal region represents a higher gradient level than the proximal (Child, 1928). In the case of the lower concentrations the pieces develop in the solution and their development is somewhat retarded at first, but when the primordia begin to appear, a few hours to a day or more later than in the controls, their development proceeds as rapidly or almost as rapidly as in the controls. These facts suggest that some degree of acclimation to these low concentrations occurs and the increases in unipolar forms indicate that this acclimation is differential, that is, that the distal region acclimates more rapidly than the proximal. Again this is to be expected if a gradient is present. But whatever the interpretation of the increases in unipolarity under the experimental conditions, it is evident that the effect of the experimental conditions on the development of the hydranth primordia is not the same in degree at the two ends of the piece.

DISCUSSION

The data presented, like earlier data, show that in pieces below a certain length the length of the distal hydranth primordium decreases with, but less rapidly than, the length of the piece. For example, the average primordium length is 60.2-micrometer divisions in the 10–12-mm. controls, 46.7 in 6–7-mm. controls, 35.25 in 4–5-mm. controls, and 28.4 in 2–3-mm. controls. Driesch (1899) regarded this decrease in primordium length as strong evidence in support of his vitalistic conception, but it appears to be merely a physiological effect of conditions at the proximal cut end on the development at the distal end which are probably of brief duration after section. In short pieces below a certain length the whole length is involved in hydranth development and undergoes reorganization. In such pieces there is apparently little or no effect of one end on the other, or the effects balance or compensate each other.

The experimental conditions considered above also decrease primordium length in pieces of all lengths used. These conditions are primarily inhibiting or retarding in their effect on primordium development and evidently affect primordium length in the same way as various physiological conditions. As already noted, the primordium does not develop by the growth in size and the differentiation of embryonic tissue arising from the cut end, but by the transformation of a region of the stem without outgrowth. The primordium does not increase in length after its first appearance. Its various parts are localized at different distances from the cut end, and the size of the hydranth at the time of emergence is determined by the scale of organization of the primordium. A change in length of primordium involves a change in the localization of the various parts with respect to the cut end, that is, a change in scale of organization. KCN and increase in hydrogen-ion concentration, as well as the physiological conditions associated with length of piece and the physiological state of the animal, bring about this change of scale. Under the experimental conditions the primordium is decreased in length and its parts are differently localized with respect to the cut end from the parts of the control primordium.

Evidently the scale of organization of the primordium is not fixed and determined by factors inherent in the protoplasm but is controlled by physiological conditions determined by relations to other parts and to external factors. The fact that in the partial forms the more distal parts of the hydranth, even the hypostome alone, develop normally in the complete absence of other parts, so far as length of piece and scale of organization permit, shows that localization and determination of parts in hydranth development take place from the apical region basipetally. The region which develops as apical end is the primary dominant region in development and is determined independently of other parts.

KCN is an extremely effective inhibitor of respiratory metabolism in protoplasms, and increase in hydrogen-ion concentration in sea water, within certain limits, usually has a similar effect to some degree. That the decrease in scale of organization of the hydranth primordium by KCN and by increase in hydrogen-ion concentration is in some way directly or indirectly associated with the effect of these conditions on metabolism is a justifiable conclusion. If it is correct, metabolism appears to be an essential factor in determining localization and scale of organization—in other words, metabolism is a real formative factor. Parker (1929, p. 424) has recently asserted that "the metabolic activity of an organism is not a true formative process but is rather the result of such a process," but has presented no evidence in support of this view. In the light of the data of experimental biology this statement is somewhat surprising. If metabolism is not a formative process how does development take place? There is a large volume of evidence showing or indicating that metabolism is a formative process, but what evidence is there to indicate that it is not such a process?

As already noted, the increase in the frequencies of wholes in the 2–3-mm. pieces under the experimental conditions is obviously correlated with the decrease in primordium length. The 2–3-mm. piece which develops as a partial form when the scale of organization is large develops as a whole when the scale is sufficiently decreased, so that hydranth, stalk, and stem are all localized within the length of the piece. The data on primordium length and on frequencies of wholes supplement and confirm each other.

And, finally, the experimental conditions increase the frequencies of unipolar forms, that is, they alter polarity as well as localization of parts in the short piece. It has been suggested above that the changes in polarity result from differential recovery after return to sea water or from differential acclimation in the lower concentrations with continuous exposure. The only other possible interpretation which suggests itself is that development at the proximal end takes place at a metabolic level nearer the developmental threshold than at the distal end, either because the developmental threshold is higher or the metabolic level lower at the proximal end or perhaps because both these conditions exist. On the basis of such a hypothesis hydranth development would be inhibited by a lesser degree of inhibition at the proximal than at the distal end. At present, however, there is no evidence to support this hypothesis and we know little or nothing about developmental thresholds, but various cases of differential recovery and differential acclimation are known and their effects on development are similar to those observed in the case of *Tubularia*. But whatever the interpretation of the facts, it is evident that the effect of the experimental conditions on hydranth development differs in degree at the two ends of the pieces concerned, and this differential effect indicates a quantitative difference in physiological condition at the two ends, that is, a gradient of some sort. Without such a difference the increase in unipolar forms would be impossible. In short, the data show beyond question that in *Tubularia* the experimental conditions to which the pieces were subjected alter localization, scale of organization, and polarity.

SUMMARY

1. KCN m/50,000, m/75,000, and m/100,000 with 48–72 hours' exposure, m/125,000 and m/150,000 with continuous exposure and sea water at pH 7, decrease the length of the hydranth primordium. In other words, these experimental conditions alter the scale of organization and the localization of the various parts with respect to the cut end.

2. In pieces 2–3 mm. in length KCN increases the frequency of wholes, that is, of forms including hydranth, stalk, and stem. This effect is correlated with the decrease in length of the primordium since the longer primordia often include the whole length of these short pieces or are even longer than such pieces, while the shorter primordia leave a part of the piece to develop as stalk and stem.

3. KCN also alters the polarity of 2–3-mm. pieces since it increases the frequency of unipolar as compared with bipolar forms. Apparently such alteration of polarity can result only from a quantitative difference in physiological condition at the two ends of the piece. It is suggested that it is due to some degree of differential recovery in the experiments with temporary exposure to KCN and to some degree of differential acclimation in those with temporary exposure.

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THE MODIFICATION OF THE NORMAL GROWTH-PROMOTING POWER, FOR PLANARIAN WORMS, OF THE DIGESTIVE MUCOSA OF THE RABBIT UNDER VARIATIONS IN DIET, FASTING, AND AGE

(Five figures)

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LAST year I (Bahrs, 1929*a*) showed that different regions of the digestive mucosa of the rabbit exhibited marked variations in growth-promoting power for planarian worms. Six regions of the digestive tract were used, as follows: the posterior half of the esophagus, the fundus of the stomach, the duodenum at the entrance of the pancreatic duct, the anterior part of the caecum, and the last part of the colon having three bands of taenia coli. When these six regions were used as the sole diets for experimental groups of planarian worms, the following increasing order in growth-promoting power was observed: esophagus and colon < stomach and caecum < duodenum < vermiform process of the caecum.

The experiments to be described in this paper are an extension of the foregoing work. The worms[1] used for all experiments were 6, 7, and 8 mm. in length at the beginning of the experiment, and were from recently regenerated tails. The experimental groups each consisted of 30 measured worms, the total length of the group being in most cases slightly over 200 mm. Each experimental group of worms was fed upon a particular region of the digestive tract of the rabbit for a period of either 3 or 4 weeks; and at the end of the experiment each worm was again measured, the total gain in length of the experimental group thus being determined. The worms were fed twice per week upon the digestive tract from freshly killed rabbits, and each part of the tissue was carefully washed in running tap water before being fed to the worms. The sections of tissue were tied up and placed in the experimental dishes in such a manner that the worms could feed only upon the digestive mucosa.

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[1] The species of planarian worm used is probably *Planaria agilis*, according to Hyman (1929).

The following experiment, in which the digestive tissue of the guinea pig was used, shows that the growth-promoting power is, however, resident only in the digestive mucosa. Two experimental groups of 30 worms each were fed in the following manner: One group received the fundus of the stomach from which the mucosa had been carefully scraped, and at the end of the experiment this group had made no gain in length but had only maintained its original length of 212 mm. The other group received the mucosa which had been scraped from the fundus of the stomach, and this group increased in length from 207 to 245 mm., a gain of 38 mm., or 18 per cent.

While working with guinea-pig digestive tissues, the lack of a vermiform process in the caecum came to my attention. This made it of interest to test the growth-promoting power of the tip of the caecum, in order to discover whether it might possess the high growth-promoting power of the vermiform process of the rabbit's caecum. Three experimental groups of 30 worms were employed, each having a total length of 180 mm. The gains in length of the experimental groups are graphically represented in Figure 1. It has been demonstrated (Bahrs, 1929*a*) that the vermiform process of the rabbit's caecum possessed a higher growth-promoting power for planarian worms than did the small intestine. Figure 1, however, shows that the growth-

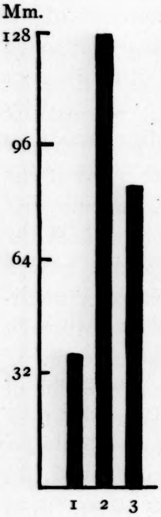

FIG. 1

FIG. 1.—Summary of Experiment I, showing that the terminal tip of the guinea-pig's caecum did not correspond, in growth-promoting power, to the vermiform process of the rabbit's caecum. Diet of guinea pigs: alfalfa hay and rolled barley. Length of experiment: 6 weeks. Worms fed once per week upon the fresh guinea-pig digestive tract. Each column represents the gain in millimeters of an experimental group of 30 worms fed upon sections of guinea-pig digestive tract, as follows:

Column 1.—Fundus of stomach; original length of worms, 180 mm.; gain, 37 mm.; gain percentage, 20.

Column 2.—Duodenum; original length of worms, 180 mm.; gain, 127 mm.; gain percentage, 70.

Column 3.—Tip of caecum; original length of worms, 180 mm.; gain, 84 mm.; gain percentage, 46.

promoting power of the tip of the guinea pig's caecum was very much lower than that of the small intestine. It would have been surprising to have found any other result, because the tip of the guinea pig's caecum is not differentiated from other parts of the caecum. In contrast, the vermiform process of the rabbit's caecum exhibits much differentiation from the caecum proper.

In all experiments in which the digestive mucosa exhibited the following order in growth-promoting power—esophagus and colon < stomach and caecum < duodenum < vermiform process of the caecum—the diet of the rabbits consisted of alfalfa hay and rolled barley. The next series of experiments was undertaken to discover whether or not the order in growth-promoting power exhibited by these six typical sections of rabbit digestive tract could be modified by the diet fed the rabbits and by fasting. Rabbits about 4 months old were used, and these were fed upon the usual diet of alfalfa hay and rolled barley until 1 week before they were to be killed for the experiment. During this week they received stale bread and a small amount of raw carrot. An excess of bread was always before the animals, and it was observed that they ate the bread. Their consumption of bread was, however, very much less than of alfalfa hay and rolled barley, when they were fed upon that diet. One group of rabbits received the bread and carrot diet up to the time that they were killed for the experiment, while another group of rabbits receiving the same diet were fasted 48 hours before being killed. In this experiment, all portions of the digestive tract were tucked in at the ends or tied up in such a manner that the worms could feed only upon the digestive mucosa. The gains in length of the experimental groups of worms are graphically represented in Figure 2.

When Experiment II, A (bread and carrot diet), is compared with Experiment II, C (normal diet), Figure 2, it is evident that the bread and carrot diet markedly affected the growth-promoting power of the vermiform process of the caecum (No. 5 in A and C, Fig. 2), reducing it 17 per cent (from 53 to 36 per cent). Reduction in growth-promoting power has also occurred in the fundus of the stomach (from 28 to 12 per cent, a 16 per cent reduction), and the duodenum (31 to 23 per cent, or an 8 per cent reduction). The caecum has also been reduced in growth-promoting power from 26 to 10 per cent, a 16 per cent reduction. When Experiment II, B, in which the rabbits were taken from the bread and carrot diet and fasted 48 hours before killing, is compared with Experiment II, C (rabbits on the normal diet), it is seen that the reduction in the growth-promoting power of the fundus of the stomach, duodenum, caecum, and vermiform process of the caecum (Nos. 2, 3, 5, of B and C, Fig. 2) has been carried still lower. The growth-promoting power

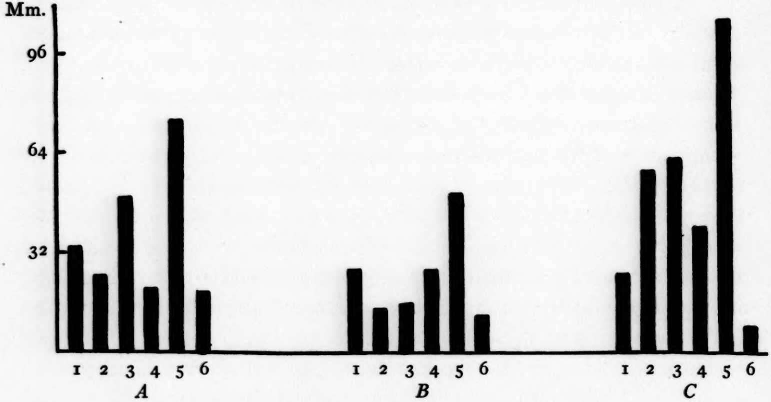

FIG. 2.—Summary of Experiment II, showing the alteration in the growth-promoting power, for planarian worms, of rabbit digestive mucosa, caused by a modification of the diet fed the rabbits. Experimental period: 3 weeks. Worms fed twice per week. Diet of rabbits as follows: Group A, bread and carrots; Group B, same but animals fasted 48 hours before killng; Group C, alfalfa hay and rolled barley.

Group A.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa of rabbits maintained upon a diet of bread and carrots. Percentage gains of experimental groups: Group 1, 16; Group 2, 12; Group 3, 23; Group 4, 10; Group 5, 36; Group 6, 9.

Group B.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed digestive mucosa of rabbits maintained upon the same diet but fasted 48 hours before killing. Percentage gains of experimental groups: Group 1, 13; Group 2, 6; Group 3, 7; Group 4, 12; Group 5, 24; Group 6, 5.

Group C.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed digestive mucosa of rabbits maintained upon a diet of alfalfa hay and rolled barley. Percentage gains of experimental groups: Group 1, 12; Group 2, 28; Group 3, 31; Group 4, 26; Group 5, 53; Group 6, 4.

The sections of digestive tract fed the experimental groups were the following: (1) esophagus, (2) fundus of stomach, (3) duodenum, (4) caecum, (5) vermiform process of caecum, (6) colon.

of these tissues was reduced in the following manner (comparing Experiments B and C, Fig. 2): fundus of the stomach, 28 to 6 per cent; duodenum, 31 to 7 per cent; caecum, 26 to 12 per cent; vermiform process of the caecum, 53 to 24 per cent. It is also of interest that the esophagus and colon which caused worms to gain 16 per cent and 9 per cent, respectively, in Experiment II, A (Fig. 2), have had their growth-promoting power reduced to 13 per cent and 5 per cent, respectively, in Experiment II, B. Fasting was thus more disastrous to the growth-promoting power of the digestive mucosa than a restricted diet.

In order to test further the effect of an inadequate diet upon the growth-promoting power of rabbit digestive tract, one group of rabbits was fed a diet of rolled barley. They received barley exclusively for 2 weeks, but after this time an allowance of about 10 gm. of alfalfa hay per rabbit was added. This allowance of alfalfa was continued until 1 week before the rabbits were to be killed for the experiment. During the last week they received only rolled barley. A second group of rabbits received the usual diet of rolled barley and alfalfa hay, to which was added a small amount of fresh cabbage or spinach. Knowing the excellent growth-promoting power of fresh liver for planarian worms (Wulzen, 1926; Bahrs, 1929*b*), it was decided to try the effect of feeding fresh beef liver to the rabbits, because it appeared that this might have an effect upon the growth-promoting power of the digestive tract. A third group of rabbits thus received alfalfa hay together with approximately the same amount of cabbage or spinach as did the control group. In addition, this group also received a mixture of 75 per cent ground rolled barley and 25 per cent fresh ground beef or pork liver. The rolled barley was reduced to the consistency of a rather fine meal by passing it through a meat chopper, and the liver was also ground to a pasty consistency. The ground liver and barley were then thoroughly blended. There was no difficulty in inducing the rabbits to eat this mixture, either in the fresh condition or after it had remained on ice for 24 hours and become slightly dry. The rabbits were placed upon these diets 2 weeks before the beginning of the feeding experiments upon their digestive mucosa. In this experiment the tissues were so arranged that the worms could feed only upon the digestive mucosa.

The total gains in length, in millimeters, of each experimental group of worms are graphically represented in Figure 3.

When Experiments III, A (diet of rabbits, rolled barley), and III, B (diet of rabbits, normal), are compared (Fig. 3), it is evident that the inadequate diet of barley alone had caused the vermiform process (No. 5 in A and B, Fig. 3) to lose nearly half of its growth-promoting power. There was also an 8 per cent reduction in the

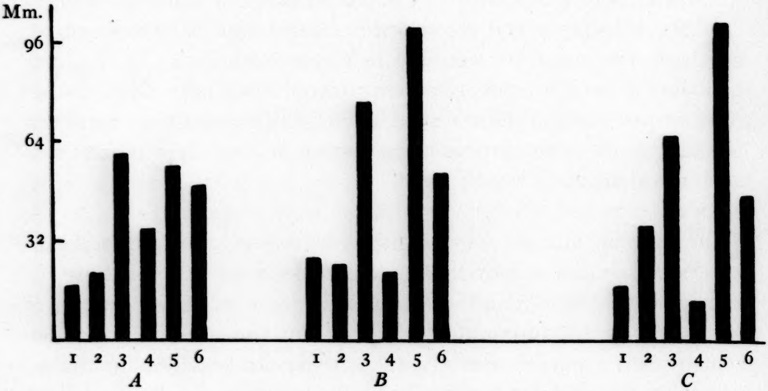

FIG. 3.—Summary of Experiment III, showing the modification in the group-promoting power, for planarian worms, of the six typical regions of rabbit digestive mucosa, due to a change in the diet fed the rabbits. Experimental period, 3 weeks; worms fed twice per week.

Group A.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa from rabbits maintained upon a diet of rolled barley. Percentage gains of experimental groups: Group 1, 8; Group 2, 10; Group 3, 27; Group 4, 16; Group 5, 25; Group 6, 23.

Group B.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa of rabbits maintained upon a diet of alfalfa hay, rolled barley, and cabbage or spinach. Percentage gains of experimental groups: Group 1, 12; Group 2, 11; Group 3, 35; Group 4, 10; Group 5, 46; Group 6, 25.

Group C.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa of rabbits maintained upon a diet of alfalfa hay, cabbage or spinach, and a mixture of 75 per cent rolled barley and 25 per cent raw liver. Percentage gains of experimental groups: Group 1, 8; Group 2, 17; Group 3, 30; Group 4, 6; Group 5, 47; Group 6, 21.

The sections of digestive tract fed the experimental groups were the following: (1) esophagus, (2) fundus of stomach, (3) duodenum, (4) caecum, (5) vermiform process of caecum, (6) colon.

growth-promoting power of the duodenum (No. 3 in A and B, Fig. 3); while the esophagus, fundus of the stomach, and colon (Nos. 1, 2, and 6, A and B, Fig. 3) also showed slight losses.

The addition of raw liver to the diet of the rabbits in Group III, C, did not increase the growth-promoting value of the digestive mucosa to the planarian worms which were fed upon it. The slight increases in growth-promoting power shown by the fundus of the stomach and the vermiform process of the caecum (Nos. 2 and 5, Fig. 3, C) over these parts of the digestive mucosa from rabbits fed upon a normal diet (Nos. 2 and 5, Fig. 3, B) are too small to be of any significance.

The experimental groups of worms which received the vermiform process of the caecum from rabbits fed the normal diet (No. 5, Fig. 3, B) and from rabbits maintained upon the liver and barley mixture (No. 5, Fig. 3, C) differed in total length at the end of the experiment by but 2 mm., or 1 per cent. As these two experiments check each other so closely, the point is established that the diet of rolled barley (Exp. III, A) was the cause of the great reduction in the growth-promoting power of the vermiform process of the caecum.

The effect of these diets upon the growth-promoting value of the livers of the rabbits, in Groups III A, B, and C, was also tested. The percentage gains of the experimental groups of worms were the following: 45 per cent for worms receiving the livers from the barley-fed rabbits; 56 per cent for worms receiving the livers from rabbits upon a diet of alfalfa hay and rolled barley; 59 per cent for the worms fed upon the livers of rabbits receiving the mixture of raw liver and barley. These results parallel those observed when the digestive mucosa of the rabbits was examined. When rabbits were maintained upon an inadequate diet, as Experiments II and III show, the growth-promoting power of the digestive mucosa, especially that of the vermiform process of the caecum, was reduced. Fasting the rabbits after they had been maintained upon a limited diet was found to increase the loss in growth-promoting power due to the inadequate diet alone. (Compare A, B, and C of Fig. 2.)

The next experiment was an attempt to determine whether the fasting of rabbits 48 hours after they had been maintained upon an optimum diet would have any effect upon the growth-promoting power of their digestive mucosa. The rabbits used for this experiment were between 2 and 3 months old. They were fed the usual diet of alfalfa hay and rolled barley, to which was added a small amount of fresh cabbage. Forty-eight hours before the worms were to receive the tissues, one rabbit was placed in a small cage and allowed to fast. (Water was given all fasting rabbits.) At the same time that this experiment was done, the effect of the age of the rabbit upon the growth-promoting power of its digestive mucosa was also tested, and the rabbits used for this experiment were all over 1 year old. Thus six experimental groups of worms received the six typical regions of the digestive mucosa from rabbits 2 months old which had been fed upon alfalfa hay, rolled barley, and cabbage but which had fasted 48 hours before being killed. Six experimental groups of worms were fed upon the digestive mucosa from rabbits 2 months old which received this diet up to the time that they were killed. Lastly, six experimental groups of worms were fed upon the same six regions of the digestive mucosa from rabbits which were over 1 year old and which received the normal diet (alfalfa hay, rolled barley, and cabbage). The total gains in length, in millimeters, of each experimental group of 30 worms are graphically represented in Figure 4. This experiment continued for 4 weeks, but measurements of length were also made at the end of the third week. These tissues were so arranged that the worms could feed only upon the digestive mucosa.

A comparison of Experiments IV, A (Fig. 4, A,) (diet of rabbits, alfalfa hay, rolled barley, and cabbage, but rabbits fasted 48 hours before killing) and IV, B (Fig. 4, B), (diet of rabbits same, not fasted) shows that the growth-promoting power of the digestive mucosa from the fasting rabbits was considerably less than that of the nonfasting rabbits. The difference in the percentage gains between experimental groups of worms fed upon the fundus of the stomach from fed and fasting rabbits (No. 2, Fig. 4, A and B) was 13 per cent in 3 weeks. In 4 weeks the difference had widened to 21 per cent, showing that this experimental group of worms (4, B, fed fundus from rabbits receiving normal diet) was growing rapidly, while the group receiving the fundus from rabbits maintained upon the same diet but fasted 48 hours before killing (4, A) was growing much more

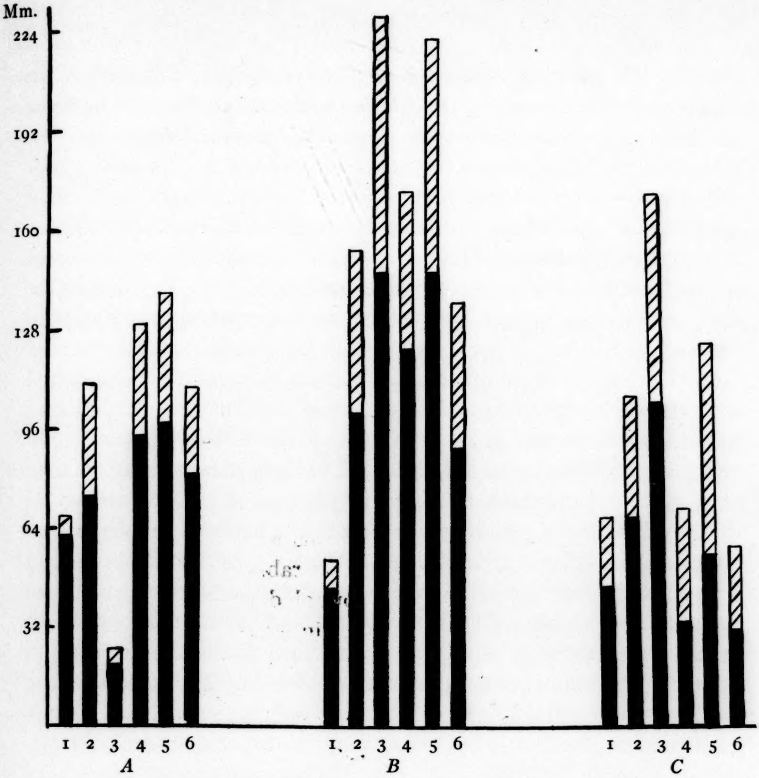

FIG. 4.—Summary of Experiment IV, showing the alteration in growth-promoting power, for planarian worms, of rabbit digestive mucosa, caused by fasting rabbits 48 hours and by age of rabbits. Diet of rabbits prior to fasting normal: alfalfa hay, rolled barley, and cabbage. Rabbits used in Experiments A and B, between 2 and 3 months old; those used in Experiment C, over 1 year old. Experimental period, 4 weeks, but measurements also made at end of third week.

Group A.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa of rabbits fasted 48 hours. Percentage gains of experimental groups: Group 1, 29–32; Group 2, 36–53; Group 3, 9–12; Group 4, 45–62; Group 5, 47–67; Group 6, 39–52.

Group B.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa of rabbits maintained upon the normal diet to the time of killing. Percentage gains of experimental groups: Group 1, 21–25; Group 2, 49–74; Group 3, 70–110; Group 4, 58–83; Group 5, 70–106; Group 6, 43–65.

Group C.—Gains in length, in millimeters, of the six experimental groups of 30 worms each fed the digestive mucosa of rabbits over 1 year old, maintained upon the normal diet. Percentage gains of experimental groups: Group 1, 22–32; Group 2, 32–51; Group 3, 50–82; Group 4, 16–34; Group 5, 26–59; Group 6, 14–27.

The sections of digestive tract fed the experimental groups of worms were the following: (1) esophagus, (2) fundus of stomach, (3) duodenum, (4) caecum, (5) vermiform process of caecum, (6) colon.

NOTE.—Black columns represent growth of experimental groups of worms in 3 weeks; shaded columns represent the growth during the fourth week, making this figure comparable with those representing other 3-week experiments. In representation of percentage gains, first figure indicates percentage gain in 3 weeks and second that in 4 weeks.

slowly. The greatest difference in growth-promoting power of the digestive mucosa from fed and fasting rabbits was observed between the two samples of duodenum. The experimental group of worms which received the duodenum from rabbits fed a diet of alfalfa hay, rolled barley, and cabbage had in 3 weeks a total length 61 per cent greater than that of the similar group which was fed the duodenum from rabbits maintained upon the same diet but fasted 48 hours before killing. (Compare No. 3 of A and B, Fig. 4.) In 4 weeks the difference in total length between these two experimental groups of worms had become 98 per cent. During the fourth week, the experimental group of worms fed upon the duodenum from the fasted rabbits increased its length by 3 per cent; but in the same period of time the experimental group of worms fed upon the duodenum from non-fasted rabbits increased its length by 40 per cent, thus growing over thirteen times as rapidly.

The difference in growth-promoting power between the vermiform process of the caecum from the fed and fasting rabbits came next in magnitude to the great difference shown in the samples of duodenum from the two groups of rabbits. By the end of the third week the experimental group of worms fed vermiform process of the caecum from rabbits maintained upon the normal diet had exceeded the similar experimental group, which received the vermiform process from the fasted rabbits, by 23 per cent (No. 5 of A and B, Fig. 4); and in 4 weeks the difference in length between the two groups of worms had become 39 per cent. A comparison of the growth-promoting power of the caecum from the rabbits maintained upon the normal diet and the caecum from rabbits receiving the same diet but which had fasted 48 hours before killing showed that this tissue had also suffered a loss. The experimental group of worms which received the caecum from the fed rabbits exhibited in 3 weeks a gain in total length which was 13 per cent greater than that of the similar group fed upon the caecum from rabbits maintained upon the same diet but fasted 48 hours before killing. (Compare No. 4 of A and B, Fig. 4.) The difference in total length between the two experimental groups of worms had become 21 per cent by the end of the fourth week. Comparing the growth-promoting power of the colon (No. 6 of A and B, Fig. 4) from the two groups of rabbits, the experimental group of worms which received colon from the fed rabbits had a total length 4 per cent greater at the end of 3 weeks than the experimental group of worms which received colon from the fasting rabbits. In 4 weeks the difference between the two groups of worms had become 13 per cent.

That fasting had had a deleterious action upon the growth-promoting power of the fundus of the stomach, duodenum, caecum, vermiform process of the caecum, and colon was emphasized by the fact that the differences between the experimental groups fed the normal and fasting rabbit tissues were greater by the end of the fourth week. This showed that the experimental groups of worms receiving these tissues from the fasted rabbits were growing much more slowly than the group receiving the same tissues from the rabbits fed up to the time of killing.

The results obtained when the digestive mucosa from rabbits over 1 year old was fed to planarian worms make it evident that the fundus of the stomach, duodenum, caecum, vermiform process of the caecum, and colon all have a lower growth-promoting power than the same tissues from young rabbits. The differences in growth between worms which received the digestive mucosa from old and young rabbits were as follows (see Experiments B and C, Fig. 4): fundus of the stomach, 17 per cent in 3 weeks and 23 per cent in 4 weeks; duodenum, 20 per cent in 3 weeks and 28 per cent in 4 weeks; caecum, 42 per cent in 3 weeks and 49 per cent in 4 weeks; vermiform process of the caecum, 44 per cent in 3 weeks and 47 per cent in 4 weeks; colon, 29 per cent in 3 weeks and 38 per cent in 4 weeks. In this experiment also, the differences in growth between the experimenal groups of worms fed the digestive mucosa from old and young rabbits became greater as the experiment progressed, demonstrating more clearly the fundamental differences in growth-promoting power between the digestive mucosa from these two groups of rabbits.

The livers from the young fasting rabbits, young fed rabbits, and old fed rabbits also showed differences in capacity to promote growth in planarian worms, in harmony with the results of the experiments upon the digestive mucosa. The experimental group of worms which received liver from the fed rabbits was 14 per cent longer by the end of the third week than the group which received liver from the fasting rabbits, and this difference was maintained through the fourth week. By the end of the third week, the experimental group of worms which received liver from the young fed rabbits had a total length greater by 22 per cent than the experimental group maintained upon liver from the old rabbits, but by the end of the fourth week this difference had dropped to 11 per cent. These results show that the factors of fasting and of age, which produced a deleterious action upon the growth-promoting power of the digestive mucosa of the rabbit also had the same effect, but considerably less, upon the growth-promoting power of the liver.

DISCUSSION AND CONCLUSIONS

It is of interest that in both of the experiments in which the rabbits were fed an inadequate diet (Exps. II, A, and III, A) the region of the digestive mucosa which suffered the greatest loss in growth-promoting power was the vermiform process of the caecum. In one of the experiments in which the rabbits were fasted 48 hours before killing (Exp. II, B; diet, bread and carrots) the vermiform process of the caecum again showed the greatest loss in growth-promoting power. When the rabbits were maintained upon an optimal diet and fasted 48 hours before killing (Exp. IV, A), the duodenum exhibited the greatest loss in growth-promoting power; but the vermiform process of the caecum came next in order. In the experiment in which the effect of the age of the rabbits upon the growth-promoting power of the digestive mucosa was tested, the loss in the growth-promoting power of the vermiform process of the caecum was the greatest. Thus, of the five experiments in which the characteristic growth-promoting power of the six typical regions of the digestive mucosa was reduced, in four the region which showed the greatest loss was the vermiform process of the caecum, and in the one experiment in which the vermiform process of the caecum did not exhibit the greatest loss in growth-promoting power it ranked second in order.

Although at present unexplainable, variations in the growth-promoting power of different regions of the digestive mucosa from rabbits fed upon an adequate diet have been demonstrated. These variations may be expressed in the following increasing order: esophagus and colon < stomach and caecum < duodenum < vermiform process of the caecum. Figure 5 shows the histogram obtained when average figures are plotted for these six regions of the digestive mucosa from all the experiments in which the rabbits received adequate diets (alfalfa hay, rolled barley, with or without raw cabbage).

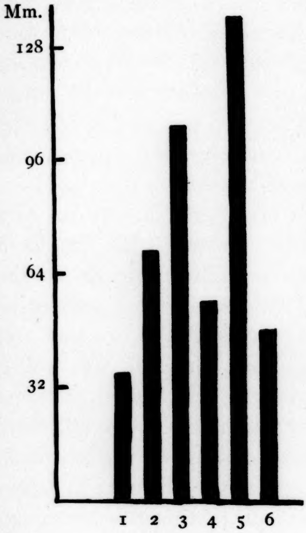

FIG. 5

FIG. 5.—Showing average growth-promoting power for planarian worms of the six typical regions of rabbit digestive mucosa, taken from all the experiments reported in which the rabbits received the normal diet of rolled barley and alfalfa hay (with or without green food, such as cabbage or spinach). Each column represents the average gain in length, in millimeters, during 3 weeks, of an experimental group of 30 planarian worms fed as indicated:

Column 1.—Esophagus (average from six experiments).

Column 2.—Fundus of stomach (average from six experiments).

Column 3.—Duodenum (average from six experiments).

Column 4.—Caecum (average from five experiments).

Column 5.—Vermiform process of caecum (average from six experiments).

Column 6.—Colon (average from five experiments).

SUMMARY

1. In all experiments, each portion of the digestive mucosa was fed to an experimental group of 30 planarian worms, and the total number of worms used in these experiments was 2,730.

2. When rabbits were fed a normal diet of alfalfa hay and rolled barley, with or without green food, such as cabbage or spinach, the six typical regions of the digestive tract exhibited the following order in growth-promoting power for planarian worms: esophagus and colon < stomach and caecum < small intestine < vermiform process of the caecum.

3. The growth-promoting power was found to be present in the

mucosa of the digestive tract, as worms fed upon the smooth muscle did not grow.

4. The growth-promoting power of the digestive mucosa was modified by the diet of the rabbits: (*a*) When rabbits were fed the inadequate diet of bread and old, raw carrots, the growth-promoting power of the fundus of the stomach, duodenum, caecum, and vermiform process of the caecum were reduced, the reduction being greatest in the vermiform process of the caecum. (*b*) When rabbits were fed the limited diet of rolled barley, a loss in the growth-promoting power of the duodenum and vermiform process of the caecum resulted, the loss being greater in the vermiform process of the caecum.

5. The growth-promoting power of the digestive mucosa was modified by fasting: (*a*) Fasting rabbits 48 hours after they had been maintained upon the inadequate diet of bread and carrots caused a much greater loss in the growth-promoting power of the fundus of the stomach, duodenum, caecum, and vermiform process of the caecum than did the inadequate diet. The loss in growth-promoting power was greatest in the vermiform process of the caecum. (*b*) When rabbits had been maintained upon an adequate diet (rolled barley, alfalfa hay, and cabbage or spinach), and fasted 48 hours before killing, losses in the growth-promoting power of the following regions of the digestive mucosa resulted: fundus of the stomach, duodenum, caecum, and vermiform process of the caecum. The duodenum suffered the greatest loss in growth-promoting power through this fast, but the vermiform process of the caecum was next in order.

6. The growth-promoting power of the digestive mucosa was modified by the age of the rabbits. The six typical regions of the digestive mucosa from rabbits over 1 year old were fed to experimental groups of planarian worms; and when the results were compared with control groups fed the same tissues from rabbits between 2 and 3 months of age, it was evident that the growth-promoting power of the fundus of the stomach, duodenum, caecum, and vermiform process of the caecum had suffered a great loss. The loss in growth-promoting power was greatest in the vermiform process of the caecum.

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UNBALANCE IN PLANARIAN NUTRITION[1]

(Three figures)

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DURING the course of experiments upon the growth-promoting power for planarian worms of blended liver pulp, egg white, and starch paste, we observed that certain experimental groups developed a pathological condition which appeared to be of nutritional origin. Single or multiple swellings, having the appearance of clear blisters or cysts, arose in the integument and often carried on their surface fragments of pigmented epidermis. The epidermis was also frayed around the base of the blisters. The most frequent type was a single cyst which involved the whole posterior extremity and gave a ball-like appearance to the tail of the worm. This ultimately swelled to the bursting-point and left the anterior portion of the worm with an excoriated surface which might heal but which more often progressed in disintegration until the entire worm disappeared. Sometimes the seat of the cyst was the anterior extremity, and all anatomical features of the head were obliterated in a mass of cytolyzing tissue. (See Fig. 1, *A*, *B*, *C*.)

In order to find the dietary limits for the development of the disease, series of experiments were constructed using diets composed of graded proportions of liver pulp, egg white, and 10 per cent starch paste. The liver was from freshly killed rabbits and was reduced to a very finely divided *brei* or pulp by pressing through heavy, close-meshed linen cloth; and the eggs used were never more than a day old. The weighed amounts of liver, egg white, and starch paste constituting the various diets were blended very carefully before being fed to the worms. The food was left in the experimental dishes for approximately 4 hours and was then removed, and the dishes were carefully washed with several changes of water. In the intervals between feeding the worms were kept clean by frequent washings. Sterilized river water and sterilized dishes were used

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throughout these experiments. The worms used (*Planaria agilis*) were 6, 7, and 8 mm. in length and were newly regenerated from tails cut from the stock worms. Each experimental group consisted of 30 worms. These were kept at an incubator temperature of approximately 24° C., were fed twice a week, and the experiments were continued for 4 weeks; in a very few cases the experimental period was 3 weeks. The total length of each group was found at the beginning of the experiment and again at the end, and the difference in total length marked the amount of growth due to the experimental diet. In this investigation, however, growth was not the main consideration.

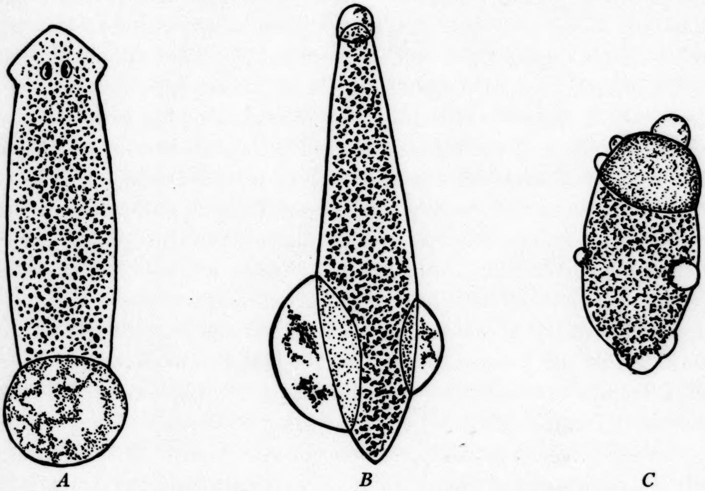

FIG. 1.—Planarian worms disintegrating under the influence of unbalance between the three elements of the diet—liver, egg white, and starch. *A*, a worm with large cyst at posterior end. *B*, a worm with anterior end much reduced in size and inclosed in small cyst; all anatomical features of normal head gone. Large cysts laterally placed on posterior end. *C*, disintegrating fragment of a worm.

As a control a graded series of experiments upon a diet of liver pulp and 10 per cent starch paste was performed. The percentages of liver pulp used were 1, 5, 10, 12.5, 15, 17.5, 20, 25, 50, and in each case the liver pulp was made up to 100 per cent with starch paste.

In the nine experimental groups fed upon these mixtures not one worm developed the pathological condition under investigation, and the groups showed a steady increase in growth with increasing amounts of liver throughout the series.

Egg white at the 50 per cent level was the basis of one experimental series. Liver pulp was added to this in proportions as follows: 0.5, 1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 22.5, 25. Each mixture was made up to 100 per cent with starch paste and was fed to a group of 30 worms during the 4-week experimental period. When the experiment had been in progress for 2 weeks, the pathological condition made its appearance in a group of worms at the center of the series, although all the other groups of the series were at that time free from the disease. At the end of the 4-week interval, the disease had developed progressively toward the ends of the series in the intermediate groups but was entirely absent from the groups at the 0.5 per cent liver level, one extreme of the series, and the 22.5 and 25 per cent liver levels, the other extreme of the series. There was an increase in the number of worms affected and the severity of the condition as the proportions of liver increased and decreased from the extremes of the series, and the maximum development of the trouble was in the group fed a combination of 50 per cent egg white, 7.5 per cent liver pulp, and 42.5 per cent starch paste. In this group only 15 worms out of the original 30 were intact at the conclusion of the experiment, and almost all of the 15 diseased worms had entirely disintegrated.

Egg white at the 30 per cent level was selected as the basis for another experimental series. The proportions of liver added to this were 1, 5, 10, 20, 25; and all mixtures were made to 100 per cent with starch paste. Here also the disease appeared after 2 weeks, and at the conclusion of the 4-week experiment the experimental groups at the extremes of the series were entirely normal while the intermediate groups were all affected, and the disease reached its maximum at the 10 per cent liver level. In this group only 15 worms out of 30 were intact at the conclusion of the experiment.

A third series was arranged upon the basis of liver at the 15 per cent level. To the liver were added graded proportions of egg white as follows: 2.5, 5, 7.5, 10, 20, 30, 40, 50, 60, 70, 85. All mixtures were made to 100 per cent with starch paste. At the conclusion of the 4-week experiment it was again found that the pathological condition had developed in the central portion of the series and that the extremes of the series showed the worms to be in a normal condition. The maximum development of the disease was in the group fed 15 per cent liver, 30 per cent egg white, and 55 per cent starch. Here only 11 worms could be measured at the conclusion of the experiment, the others being wholly or partly disintegrated.

When these results were plotted with percentages of liver and egg white as co-ordinates, it was observed that they mapped out a distinct area of food combinations which were capable of producing disease in the worms. Therefore more experiments were arranged in which groups of worms were fed those combinations of egg white, liver, and starch paste which would best complete and limit the area of harmful effect already roughly outlined.

Table I gives the results of 74 experiments, each involving the use of 30 worms, the total number of worms represented being 2,220. Duplicate experiments were performed in a number of cases and gave results in close harmony with the original experiments. The percentages of egg white (A), liver (L), and 10 per cent starch paste (S) in each food mixture are given; and the number of worms out of 30 (W) remaining normal at the conclusion of the experiment is stated.

Inspection of the list reveals that apparently slight change in percentage composition often makes large change in the toxic property of the food combinations. For instance, at the level A 10 per cent, a change from 10 to 15 per cent liver reduced the number of worms from the normal number, 30, to 15; while at the level A 30 per cent, a change from 1 to 5 per cent liver reduced the number of worms from normal to 16. On the other hand, at the level A 60 per cent, changing the liver percentage from 5 to 10 increased the number of worms from 20 to 29, practically normal. The level A 70 per cent is worthy of note. While 70 per cent A+30 per cent S gave a result of moderate damage, 25 worms remaining, when 1 per cent liver was added the damage became quite heavy, 11 worms only surviving the experiment. But from that point the addition of graded amounts of liver had an increasing power of protection, until with the combination 70 per cent A+20 per cent L the result was practically normal. At the level A 80 per cent, the combination 80 per cent A plus 20 per cent S had extreme toxic effect and the whole experiment was almost entirely wiped out. The same was true when 1 or 5 per cent liver was added, but with the addition of 10 per cent liver there was a remarkable change. In this case all the worms could be measured at the conclusion of the experiment; but, as a few were deformed in outline, the experiment was listed as slightly damaged. The level A 90 per cent showed the same violent effect

TABLE I

A	L	S	W	A	L	S	W
2.5	20	77.5	30	50	5	45	17
2.5	15	82.5	29	50	7.5	42.5	15
5	10	85	30	50	10	40	26
5	15	80	29	50	12.5	37.5	18
5	20	75	29	50	15	35	20
5	25	70	29	50	17.5	32.5	25
7.5	5	87.5	30	50	20	30	28
7.5	10	82.5	27	50	22.5	27.5	29
7.5	15	77.5	24	50	25	25	30
7.5	20	72.5	28	60	1	39	25
10	5	85	30	60	5	35	20
10	10	80	30	60	10	30	29
10	15	75	15	60	15	25	29
10	20	70	27	60	20	20	30
10	25	65	30	70	0	30	25
20	1	79	30	70	1	29	11
20	5	75	29	70	5	25	16
20	10	70	21	70	10	20	26
20	15	65	16	70	15	15	28
20	20	60	30	70	20	10	29
20	25	55	30	80	0	20	3
30	1	69	30	80	1	19	8
30	5	65	16	80	5	15	6
30	10	60	15	80	10	10	30
30	15	55	11	80	15	5	29
30	20	50	23	80	20	0	30
30	25	45	30	90	0	10	5
40	1	59	29	90	1	9	3
40	5	55	11	90	5	5	19
40	10	50	21	90	10	0	30
40	15	45	11	100	0	0	17
40	20	40	30	0	1	99	30
40	25	35	30	0	5	95	30
50	0	50	30	0	10	90	30
50	0.5	49.5	30	0	15	85	30
50	1	49	26	0	20	80	30
50	2.5	47.5	26	0	50	50	30

of high concentrations of egg white either with starch alone or with starch and 1 per cent liver. But 5 per cent liver gave a decided protective effect, and with 90 per cent A plus 10 per cent L the worms were uninjured.

In all the foregoing cases the percentage of starch was changing as well as the liver, and this change probably had a share in the total effect. For instance, when egg white was the sole element in the diet, the damage was indeed heavy, 17 worms remaining at the end of the experiment; but the damage was decidedly less than when 90 per cent A plus 10 per cent S, or 80 per cent A plus 20 per cent S, were fed. On the other hand, the next step downward, 70 per cent A plus 30 per cent S, produced an effect of moderate damage only, as 25 worms survived the experiment.

The results recorded in the list of experiments are represented graphically in two charts. In Figure 2 each experiment is registered as a block marked off on the chart, and the proportions of liver and egg white occurring in any experimental diet can be read off from the co-ordinates, while the amount of starch paste in the diet can be easily calculated. The white blocks represent experiments in which the diet produced no harmful effect on the worms. The white blocks containing a cross represent experiments in which the harmful effect of the diet was exceedingly light, only 1 or 2 worms being at all affected. The gray blocks show a moderate degree of damage, the number of worms which remained normal being 20 to 27; while in the black blocks the damage was heavy, only 3 to 19 worms being intact at the conclusion of the experiment.

In general the chart shows an elongated area in which the various blocks represent combinations of egg white, liver, and starch paste which have disease-producing power for the worms. Except for a small space where the concentrations of egg white are very high, this area is surrounded on all sides by a field of combinations of the three elements of the diet which are essentially harmless to the worms. The disease-producing area itself presents an inner zone of heavy damage incased in an outer zone of moderate and light damage. Like the harmless region, this zone of moderate damage makes way for heavy damage when the concentrations of egg white are very high.

The elongated, disease-producing area is so arranged with reference to the liver and albumin axes of the chart as to express the following relationships. Exceedingly high concentration of egg white is damaging either alone or with the admixture of very small amounts of starch and liver; but, as the percentage of egg white falls, increasingly larger amounts of liver and of starch paste must be added to the mixture to insure the continuation of the area of heavy damage. However, when the amounts of egg white become very small, the harm produced by the proper amounts of liver and starch, though undoubtedly present, is slight. Again, if one selects

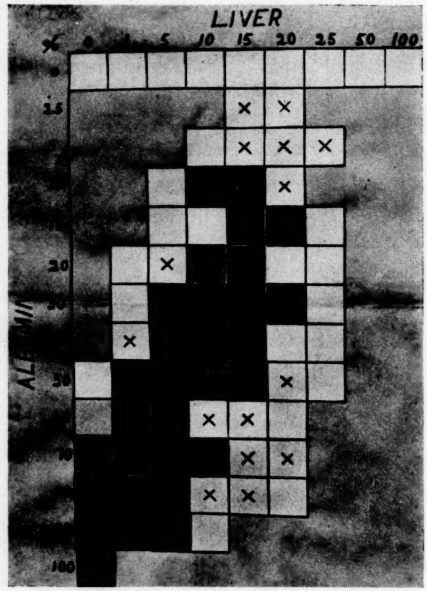

FIG. 2.—Showing combinations of liver and egg white used in experimental diets, and the results of these diets upon groups of planarian worms. Co-ordinates represent percentages of liver and albumin (egg white) in diets.

Results on the various diets are represented by blocks.

White blocks = experiments in which the diets proved harmless to the worms.

White blocks containing cross = experiments in which the diets produced very slight damage.

Gray blocks = experiments in which the diets produced moderate damage.

Black blocks = experiments in which the diets produced heavy damage.

a central point in the area of damage (for instance, the combination 40 per cent A plus 10 per cent L plus 50 per cent S) and travels along either axis in either direction, one comes to a zone of safety. In other words, the worms can be protected either by increasing or decreasing egg white if the liver remains constant, or by increasing or decreasing liver if the egg white remains constant.

In the second chart, Figure 3, the relation of the liver, starch, and egg-white axes to the axis of the area of damage is shown. Here percentages of egg white in the diet increase from the top to the bottom of the chart, percentages of liver from the left to the right, and percentages of starch increase from the bottom to the top on the oblique lines. The heavy line labeled 5 S: 1 L, which crosses the diagram and cuts the liver, starch, and egg-white co-ordinates, represents all values of starch paste and liver which are in the ratio 5:1. The area of moderate and heavy damage is shaded into the diagram as nearly as possible in its proper place; and it will be seen that the line 5 S: 1 L makes at least an acceptable axis for the area of damage. Approximately equal amounts of the area of damage appear on either side of the 5 S: 1 L axis. There is an inverse relationship between the axis of the area of damage and the egg-white axis. As the amount of egg white decreases, the amounts of liver and starch paste which must be added to the egg white to produce injury increase, and the amount of starch paste and liver necessary to produce this result bear a relationship to one another, the mean value of which is 5 S: 1 L. That is, for the production of damage there would appear to be necessary very high concentrations of egg white; or, if egg white is steadily decreased, there must be added more and more of 10 per cent starch paste and liver, balanced on the mean in the proportions 5 S: 1L.

The question arises whether the smaller amounts of starch paste and liver which produce damage with the higher amounts of egg white would be effective in producing injury if they were applied to lower concentrations of egg white. As the experiments are arranged, that question cannot be answered, because in order to apply small amounts of balanced starch and liver to low proportions of egg white, the use of some fourth material as a vehicle would be required. However, it may be noted by referring to Figure 2 that, when the amounts of egg white are very small, large amounts of the starch and liver mixture give only light or moderate damage. It might perhaps be inferred that small amounts of starch and liver would have no effect whatever.

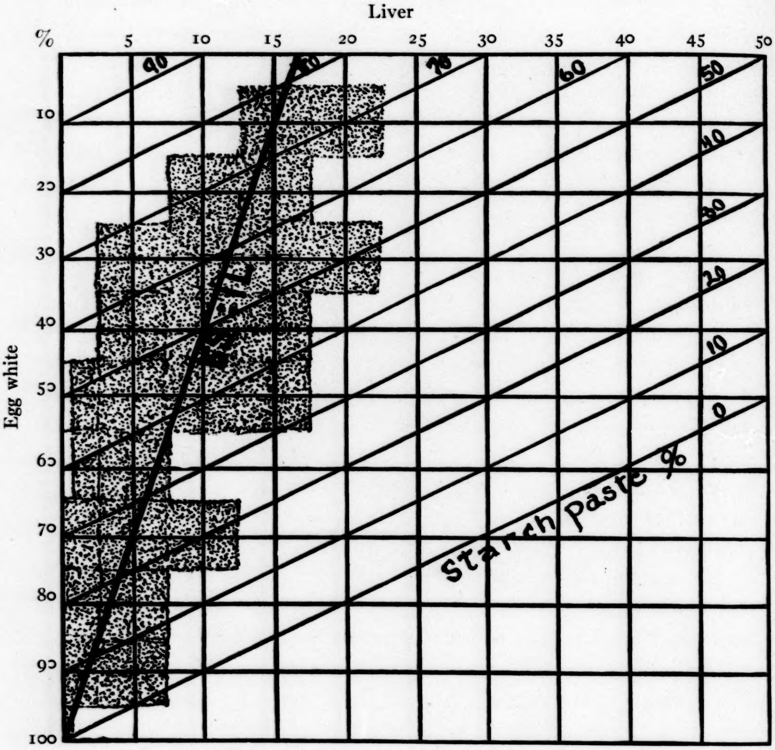

FIG. 3.—Showing the location of the area of damage with reference to co-ordinates giving percentages of liver, egg white, and 10 per cent starch paste in the experimental diets. Shaded portion represents area in which diets produce moderate or heavy damage in groups of planarian worms. The axis of the area of damage is approximately marked by the line 5 S: 1 L, which represents combinations of starch paste and liver in the ratio of 5: 1.

The actual amounts of the different food mixtures taken by the worms must obviously remain an open question. The statement may be made, however, that the worms ate every food mixture with readiness; and the fact that the food had been taken was easily ascertained from the distended shape of the worms and their whitish color after a meal. The appearance of the typical disease was proof that the food had been taken, because the worms can be fasted indefinitely with no pathological manifestations. The worms were strongly attracted by both egg white and liver, and the only question of food consumption might be where the percentages of both of these were very low. Almost no such combinations are listed.

At present it is difficult to analyze the meaning of the unbalance here demonstrated between the three constituents of the diet—egg white, liver, and starch paste. Whether the harmful combinations of these substances owe their effects to faulty protein adjustment, or to an excess or lack of some vitamin, or to some other cause, cannot at present be stated; but, through experiments which are projected for the immediate future, we hope to be able to throw some light on the whole problem.

SUMMARY

1. When planarian worms are fed diets consisting of certain proportions of egg white, liver, and starch, they develop a pathological condition characterized by a definite syndrome. Other proportions of the same food substances are entirely harmless for the worms. Also, fasting develops no such syndrome.

2. These disease-producing combinations of egg white, liver, and starch bear a definite relationship to one another so that if they are arranged according to percentages of each used, the diets are found to map out a single area within which all food combinations are damaging to the worms and outside of which all combinations are harmless.

3. When represented on co-ordinates showing percentages of liver, starch, and egg white, the area of damage is found to be so arranged that with increasing percentages of egg white there must be a decreasing percentage of liver and starch in order to secure damage; and, conversely, with increasing percentages of liver and starch, the percentages of egg white necessary to produce damage must decrease.

4. The area of damage also indicates in its structure a possible constant relationship between the amount of starch paste and the amount of liver in the diets which have disease-producing power. The approximate longitudinal axis of the area of damage represents concentrations of 10 per cent starch paste to liver in the ratio 5:1.

THE RELATION OF NUMBERS OF ANIMALS TO SURVIVAL IN TOXIC CONCENTRATIONS OF ELECTROLYTES[1]

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I. INTRODUCTION

THE harmful effects of crowding animals into a limited environment have long been known. The literature on this subject is too extensive for detailed review at this time. The development of the present-day information and point of view may be traced briefly as follows: Hogg (1854) explained such harmful effects among snails as due to their ability to adapt themselves to the necessities of their environment. Semper (1874) thought the ill effects of crowding were due to a lack of sufficient growth-producing substance in small volumes of water. Yung (1878) explained them among tadpoles as due to a lack of aëration. De Varigny (1894) concluded that a lack of exercise was the causal factor for dwarfing among snails. Vernon (1895), using echinoderm larvae; Warren (1900), using *Daphnia;* Woodruff (1911, 1914), using different Protozoa, all concluded that these harmful effects were due to accumulation of waste products in small volumes. Colton (1908) thought food and oxygen as well as waste products were influencing factors among snails. Bilski (1921) concluded that these effects among tadpoles were caused by excessive expenditure of energy on account of contact stimulations; Goetsch (1924) confirmed these results for rapidly moving animals. Pearl and Surface (1909) suggest a psychological factor to explain crowding phenomena among chickens. Peebles (1929), using sea-urchin and starfish larvae, found evidence of a growth-inhibiting substance which is of a lipoid nature and soluble in acetone. This literature is reviewed in greater detail by Allee (1931).

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[1] I wish to express my sincere thanks to Dr. W. C. Allee for suggesting this problem and for his kindly counsel throughout the work upon it. I also wish to express my thanks to Dr. Sewall Wright for valuable advice as to statistical treatment of the data.

Drzewina and Bohn (1921*c*) found that *Polycelis* did not survive as long in groups as they did as single individuals when placed in 0.03 N. KCl solution. They postulated an autodestructive secretion to account for this. Later (1925) they explained this autodestructive effect as being due to low pH brought about by cell cytolysis which accelerates further cell cytolysis.

The beneficial effects of crowding a number of animals into a limited volume when the environment is uncongenial was overlooked until about ten years ago, when Allee (1920) reported that land isopods, water isopods, and brittle starfish, when aggregated, respired more slowly and remained quieter, and under extreme conditions survived longer, than single individuals in the same environment. Drzewina and Bohn later in the same year published results which show that *Convoluta*, a marine flat worm, will survive much longer as groups than as single individuals when placed in dilute sea water. Since that time, there has accumulated considerable literature on this subject, which on the whole shows that, other conditions being equal, groups of animals may have a marked advantage in survival time over single individuals when placed in small volumes of toxic media. There have been several theories advanced at different times and under different conditions to account for this group protection against toxic media.

Drzewina and Bohn (1921*a*, *b*, *c*), experimenting with Infusoria, *Hydra*, *Convoluta*, and tadpoles of *Rana fusca* in toxic solutions of colloidal silver, potassium chloride, and fresh water, found markedly increased survival when animals were grouped over that of single individuals when exposed to the same concentration and the same volume of the toxic solution. In order to explain this phenomenon, they postulated an autoprotective secretion which they suggested is a substance (or substances) secreted by the animals which destroys the deleterious effects of the toxic solution.

Bresslau (1922, 1924) studied the effects of different dyes—methylene blue, Victorian blue, neutral red, India ink, etc.—on different Infusoria, such as *Colpidium*, *Paramecium*, etc., and found that groups of animals in a small volume of the weak dye solution survived much better than single individuals in the same volume and concentration. He accounted for this protective value of the group by the fact that these animals secrete a substance, which he calls "tektin," that forms a gelatinous hull around them and adsorbs the toxic chemical, thereby weakening its concentration and making it possible for groups to live longer than single individuals.

Allee and Schuett (1927) experimented with *Planaria* and other fresh-water animals and with *Ophioderma* in toxic solutions of colloidal silver and found marked group protection. They explained their results on the basis that these animals secrete a slime which adsorbed the silver and weakened the concentration of the medium, thus permitting the groups to survive much longer than single individuals. Their results show that this protection is not specific in effect.

Allee (1927*a*) experimented with *Ophioderma* and found that groups survived better than single individuals when under adverse laboratory conditions. When glass rods were placed in the vessel with single individuals, the individuals survived better than other individuals in the absence of glass rods. He concluded that the survival advantage of the group was at least partly due to members of the group substituting each other for objects of the normal environment, which regulated the general body tonus and made it possible for the groups to survive better than single individuals.

Allee (1929*a*) experimented with *Procerodes* in hypotonic sea water in which the electrolytes were controlled, and found that when the hypotonic sea water was conditioned with extracts of the same and of different species the animals survived significantly longer than they did in unconditioned hypotonic sea water with the same strength of electrolytes.

Drzewina and Bohn (1928), in their summarizing paper, "Les *Convoluta*," review the literature on the subject of mass protection and recognize the validity of the foregoing theories under different conditions and add another theory, which they call "catalysis by contact." This is an expression of the changes in electrical conditions produced by a group, as contrasted with those found when single animals are isolated, and is concerned with interrelations between animals as well as relations between individuals and the walls of their container.

For an interpretation of our data and an understanding of the point of view developed in the present paper, attention also needs to be called to the work of Child and his collaborators, who have repeatedly demonstrated that the animal or part of an animal which has the highest metabolic rate is the most susceptible to a great variety of injurious agents, both chemical and physical. They have shown this to be true for a large variety of animals and plants. Also, if these injurious agents are not too fatal, so that there is some acclimation, the organism or part of the organism with the highest metabolic rate will acclimate most rapidly and survive longer than one with slower metabolic rate. He calls the former "differential susceptibility" and the latter "differential acclimation." Dr. Child reviews the literature and discusses this subject in some detail in these books: *Senescence and Rejuvenescence* (1915), p. 92; *Individuality in Organisms* (1915), p. 50; *Physiological Foundations of Behavior* (1924), p. 76; also in "The Physiological Gradients," *Protoplasma*, Vol. V (1929), No. 3.

According to Child's theory, if groups of animals have a slower rate of metabolism than single individuals, then differential susceptibility between the slower metabolizing animals of the group and the singles with a higher metabolic rate may play an important rôle in the survival of groups over single individuals in highly toxic solutions. Also, differential acclimation should be effective in weaker solutions and give such singles advantage over the groups. This will be discussed in more detail below.

Fowler (1927) used *Daphnia pulex* de Geer and tested for group protection at different concentrations in the following solutions: calcium chloride, calcium nitrate, magnesium chloride, magnesium nitrate, zinc chloride, zinc nitrate, potassium chloride, potassium hydroxide, potassium cyanide, iron nitrate, barium chloride, barium nitrate, lead chlorate, lead nitrate, aluminum chlorate, aluminum nitrate, aluminum sulphate, sodium chloride, sodium nitrate, sodium nitrite, sodium silicate, sodium hydroxide, nickel sulphate, nickel sulphite, caffeine, ammonium oxalate, silver nitrate, colloidal silver, colloidal sulphur, cupric sulphate, white arsenic, sulphuric acid, nitric acid, and hydrochloric acid. Experiments were made with five different concentrations of each chemical, and the experiment with each concentration was divided into three parts as follows:

1. Twenty animals were distributed, two each, in 20 cc. of the solution in small bottles.

2. Twenty animals were put in 20 cc. of the solution in one small bottle.

3. Twenty animals were put in 200 cc. of the solution in a large bottle.

The survival times were noted, and the pH was checked at the beginning and at the end of the experiments. Results show that pH is not an important factor in these experiments.

When the survival times were tested statistically, those given when 20 animals were crowded into 20 cc. of the solution showed significant group protection over the other two parts of the experiment where the volume per animal was ten times as great. This group protection was consistent at certain concentrations throughout the foregoing list of chemicals. These preliminary experiments did not give us information as to the nature of this group protection. The following experiments are concerned with such an analysis of this phenomenon of group protection of *Daphnia*. They further give the results of an examination concerning whether or not group protection is related to the less widely observed phenomenon of autodestruction as outlined by Drzewina and Bohn. These experiments fall naturally into several groups as different methods are used. Each group of experiments will be described separately, but general discussion of the results will be reserved until all the experiments have been reviewed.

II. EXPERIMENTAL RESULTS

Daphnia longispina were used in all of the following experiments. The chemical solutions were made up in Whitman Laboratory well water except in the cases of sodium hydroxide and potassium hydroxide; distilled water was used for these two chemicals.

1. *Do chemical changes occur as animals live in the solution?*—It was noted above that the most marked protection occurred where the number of animals per unit volume of solution was the greatest. This suggested that animals living in a toxic solution might change

the chemical concentration of the solution so as to render it less toxic. This was tested by using an electrolyte, calcium chloride, as the toxic solution. Experiments were run with six different concentrations of the solution. Each experiment consisted of: (1) 20 animals in 20 cc. of each concentration of the solution, (2) 2 animals in 20 cc. of each concentration of the solution, (3) control solutions in which no animals were present. At the end of the experiments the concentrations of the different solutions were measured by the conductivity method of measuring electrolytes in which a conductivity cell containing the solution to be measured was connected in the circuit of a wheatstone bridge, to which was attached a vibrator; then, with head phones to determine the balancing-point, the resistance of the solution was measured in ohms. The experimental error of this method was about 2 per cent. These experiments were repeated ten times. Table I shows a summary of the results obtained. The data given there show that there was a total resistance of solutions in which groups of animals had lived of 854.9 ohms, as compared with 841.5 ohms for the solutions that contained isolated animals, which indicates that changes have occurred in the former equal to the removal of 1.5 per cent more electrolytes than in the latter. This lies within the experimental error. Further, when the resistances of these solutions are expressed in percentages, and Student's method (1925) for calculating significance of paired experi-

TABLE I

SHOWING SUMMARY OF SURVIVAL TIMES AND DIFFERENCES IN RESISTANCES PRODUCED BY *Daphnia* LIVING IN $CaCl_2$ SOLUTION

Molar Concentration of $CaCl_2$	Time in Hours Group Lived	Time in Hours Singles Lived	Resistance of Groups (Ohms)	Resistance of Singles (Ohms)	Resistance of Controls (Ohms)
0.05	62.00	40.84	194.2	191.5	187.0
0.06	51.83	26.66	169.7	168.0	162.5
0.07	26.08	12.24	148.0	148.5	148.25
0.08	11.06	9.66	135.5	125.0	128.5
0.09	9.41	6.74	118.0	117.5	117.7
0.10	5.98	4.16	89.5	91.0	92.0
Totals	166.9	100.3	854.9	841.5	835.95

ments is applied to them, the statistical probability is 0.3214, which is well within the range of random sampling and therefore has no significant value. The groups survived a total of 166.9 hours, as compared with 100.3 hours for single individuals. When Student's method is applied to the differences in survival time between groups and single animals in these experiments, the statistical probability is 0.0022. This probability would occur only twenty-two times in ten thousand trials by random sampling, a quite significant proportion.

Another series of experiments were run in exactly the same manner with the single exception that the same solutions were used over and over until the experiment had been repeated ten times. The solutions were then measured by the conductivity method. The total resistance of the solutions of the groups was 51.7 ohms, as compared with 51.6 ohms for the solutions of single animals. Again, this small difference is well within the experimental error and demonstrates that whatever group protection exists cannot be explained on the basis of a reduction of total concentration of electrolytes.

2. *Test for group protection when groups and singles are exposed to the same solution.*—Small tubes, such as were used by De Varigny and Goetsch, were made by fastening fine-mesh cheesecloth over one end of isinglass tubes about ¾ inch in diameter. These tubes were fastened together and supported on a wire frame about ¾ inch from the bottom of a large crystallization dish, which was filled with 0.072 molar solution of calcium chloride to a depth that would approximate 20 cc. in each tube. Two animals were placed in each of 20 tubes, and 20 animals were placed in each of 10 tubes. The solution in the tubes could diffuse through the cheesecloth with that of the surrounding solution. However, in order to make sure that there was complete mixing of the solution, the tubes were occasionally lifted out of the solution and then lowered back in position. This experiment was repeated four times. Groups showed some survival advantage over single individuals under these conditions, and this advantage was greater when the tubes were allowed to remain for longer intervals before lifting the tubes and thoroughly mixing the solution. This suggests secretion or excretory products as being the influencing agent in the survival advantage of the groups.

3. *Oxygen consumption in groups and single individuals.*—In these experiments on oxygen consumption four different sizes of bottles

were used. The first set ranged from 4.5 cc. to 4.6 cc.; the second from 7.0 cc. to 7.4 cc; the third from 15.9 cc. to 16.9 cc; the fourth from 29.7 cc. to 33.5 cc. These bottles were filled with 0.01 molar solution of calcium chloride. One animal and 5 animals were placed in the first set of bottles; in the second set 3 bottles held respectively 1, 2, and 10 animals; in the third set 1, 4, and 20; and in the fourth and largest set 1, 8, and 40 *Daphnia*. Each set had a control bottle without any animals. In all cases adult animals were used. The bottles were sealed with ground-glass stoppers. The experiments ran for 6 hours except in cases where an animal would die before the end of that time. In such cases the experiment was stopped and oxygen determinations were made at once; as a consequence a few of the experiments ranged between 3 and 6 hours. Most of them, however, ran the full 6 hours. The Winkler method, modified on account of the small volume of sample (Allee, 1929), was used in making oxygen determinations. Table II shows the results of experiments with the first two sizes of bottles. Experiments with the larger bottles in the third and fourth sets gave equivalent results. The amount of oxygen consumed is given in cubic millimeters per animal per hour. In the first set of bottles the average oxygen consumption so expressed is 0.764 for isolated individuals, as compared with 0.414 for the animals composing the groups; in the second set of bottles the averages for 1 animal, 2 animals, and 10 animals are respectively 0.986, 0.646, 0.434; in the third set of bottles the averages for 1, 4, and 20 animals are respectively 0.934, 0.568, 0.459; for the fourth set the averages for 1, 8, and 40 animals are respectively 1.522, 0.696, 0.536 cu. mm.

When these values are expressed in percentages and Student's method for calculating significances is applied to the different groups versus singles, the following figures of statistical probability are obtained:

		Mean Difference	Probability
First set,	singles versus 5's	0.350	0.001
Second set	singles versus 2's	.340	.024
	singles versus 10's	.552	.0005
Third set	singles versus 4's	.366	.0018
	singles versus 20's	.375	.0014
Fourth set	singles versus 8's	.916	.002
	singles versus 20's	0.986	0.001

TABLE II

SHOWING DIFFERENCES IN OXYGEN CONSUMPTION BETWEEN GROUPS OF *Daphnia* AND SINGLE INDIVIDUALS IN $CaCl_2$ SOLUTION

APPROXIMATE SIZE OF BOTTLES—4 CC.

Cc. O_2 per Liter, Controls	Cubic Millimeters of Oxygen per Hour per Animal	
	1 Animal	5 Animals
5.35	0.581	0.308
4.91	1.136	.260
7.18	1.358	.602
6.94	0.815	.512
6.94	0.768	.678
6.94	1.023	.603
6.94	0.907	.443
7.29	.684	.597
7.18	.548	.312
7.46	.361	.163
7.88	.605	.439
3.64	0.575	.849
6.25	1.085	.507
6.08	0.645	.272
6.25	0.361	0.166
Av. 6.48	0.764	0.414

APPROXIMATE SIZE OF BOTTLES—8 CC.

Cc. O_2 per Liter, Controls	Cubic Millimeters of Oxygen per Hour per Animal		
	1 Animal	2 Animals	10 Animals
5.52	0.819	0.544	0.360
5.51	0.471	.464	.419
7.19	1.927	.600	.371
7.36	1.089	.848	.733
6.80	1.334	.940	.444
7.31	1.888	.607	.649
7.36	0.260	.970	.598
7.54	0.509	.520	.461
7.71	1.190	.777	.448
7.96	0.800	.411	.301
8.04	0.652	0.520	.416
4.65	1.887	1.110	.460
5.82	0.549	0.350	.378
6.70	1.093	.677	.406
6.82	0.328	0.351	0.075
Av. 6.83	0.986	0.646	0.434

These figures are all of clearly significant value and indicate that the decrease in oxygen consumption shown by grouped as compared with single individuals is not due to chance of random sampling.

In order to avoid any criticism of the foregoing data on the basis that the oxygen consumption of a single animal was compared with the mean of the group, another set of experiments was made, using the third set of bottles in which the number of single individuals was equal to those of the group (15) animals. Then, by comparing the mean oxygen consumption of the group, which was 0.8124 cu. mm., with the mean of the singles, which was 1.3211 cu. mm. per animal per hour, and calculating the statistical significance as stated above, the figure of probability is 0.0001, which compares favorably with the probability figures just given. Accordingly, we may conclude that grouped animals use less oxygen per individual than do single individuals.

4. *Influence of unaërated conditioned solution upon oxygen consumption in single individuals.*—These experiments were arranged similarly to those in Section 3 except the solution in which singles were placed had been homotypically conditioned by having groups of *Daphnia* live in it. Also, the bottles with intermediate-sized groups were eliminated in these experiments, and tests were made with singles and large groups only. Table III shows the results of a series of seven experiments with the first and second sets of bottles. The third set of bottles gave equivalent results. The largest set of bottles was not used in these experiments. In the first set of bottles the average oxygen consumption expressed in cubic millimeters per animal per hour is 0.579 for single individuals, as compared with 0.538 for the groups; for the second set of bottles the average for single animals is 0.616, as compared with 0.575 for groups of 10 animals; in the third set of bottles the average for singles is 0.693, as compared with 0.694 for groups of 20 animals.

When these results were treated statistically by Student's method, the following figures of probability are obtained:

	Mean Difference	Probability
First set, singles versus 5's	0.041	0.570
Second set, singles versus 10's	.041	.759
Third set, singles versus 20's	0.001	0.807

These figures of probability are clearly not significant, and indicate that single individuals in conditioned solution have their rate of metabolism lowered to that of the group in raw solution. These results suggest that the difference in metabolism, as measured by oxygen consumption, between single individuals and groups of animals is due to the accumulation of waste products in the medium of the group, or that it is due to the difference in oxygen tension between the two solutions, which at the beginning of the experiments was only 66 per cent of air saturation in the conditioned solution.

TABLE III

SHOWING EFFECTS OF UNAËRATED CONDITIONED SOLUTION UPON OXYGEN CONSUMPTION IN *Daphnia*

APPROXIMATE SIZE OF BOTTLES—4 CC.

Oxygen Tension in Unaërated Conditioned Solution	1 Animal (Cubic Millimeters of Oxygen per Animal per Hour)	Oxygen Tension in Raw Solution	5 Animals (Cubic Millimeters of Oxygen per Animal per Hour)
4.76	0.787	6.23	0.447
4.15	1.058	7.28	.668
3.45	0.364	5.45	.553
4.08	.453	5.84	.649
4.26	.544	5.45	.507
4.07	.364	6.88	.577
4.36	0.453	5.31	0.363
Av. 4.16	0.579	6.06	0.538

APPROXIMATE SIZE OF BOTTLES—8 CC.

Oxygen Tension in Unaërated Conditioned Solution	1 Animal (Cubic Millimeters of Oxygen per Animal per Hour)	Oxygen Tension in Raw Solution	10 Animals (Cubic Millimeters of Oxygen per Animal per Hour)
5.90	0.512	6.31	0.554
3.25	.654	6.52	.635
3.01	.727	6.46	.608
3.68	.362	6.00	.599
4.06	.860	5.44	.490
4.01	.543	6.15	.596
4.54	0.653	5.85	0.545
Av. 4.06	0.616	6.10	0.575

5. *Influence of aërated conditioned solution upon oxygen consumption in single individuals.*—This series of experiments were run ex-

actly as were those in Section 4 except the conditioned solution was aërated before the single animals were put into it. The results are quite different from those in Section 4 and agree very closely with those in Section 3. In the first set of bottles the average oxygen consumption, expressed in cubic millimeters per animal per hour, is 0.617 for single individuals, as compared with 0.433 for the groups; for the second set of bottles the average for the single animals is 0.813, as compared with 0.512 for groups of 10 animals; for the third set of bottles the average for singles is 0.901, as compared with 0.604 for groups of 20 animals.

When these results are treated statistically by Student's method, the following figures of probability are obtained:

	Mean Difference	Probability
First set, singles versus 5's...........	0.184	0.009
Second set, singles versus 10's........	.301	.011
Third set, singles versus 20's.........	0.297	0.0098

These figures of probability are all significant. These results indicate that the substance or substances which retards metabolism in *Daphnia* when they are crowded into a small volume is of a volatile nature and is eliminated by aërating the solution, or that the increased metabolism of the singles in these aërated, conditioned solutions, as compared with those in unaërated conditioned solutions, may be due to increasing the oxygen tension. In these experiments the oxygen tension of the conditioned solution was about 80 per cent of air saturation.

6. *Influence of reduced oxygen tension upon the rate of oxygen consumption in Daphnia.*—A series of 10 experiments were made to determine the effects of reduced oxygen tension on the rate of oxygen consumption in *Daphnia;* 20 cc. bottles were used in these experiments. In one part of each experiment 5 animals were put in each bottle with oxygen tension at air saturation in 0.01 molar solution of calcium chloride. In the second part of the experiment 5 animals were put in each bottle of the same solution which had its oxygen tension reduced to about 50 per cent of air saturation by bubbling nitrogen through it. The experiments ran for 6 hours, at

the end of which time oxygen determinations were made with the modified Winkler method. Table IV shows the oxygen tensions at the beginning and at the end of the experiments, with the amount of oxygen consumed per animal per hour during the experiments. On the average, the animals in the solution with oxygen tension at air saturation consumed 0.758 cu. mm. per animal per hour, as compared with 0.682 cu. mm for the animals living in the same solution with oxygen tension reduced to 50 per cent of air saturation. When the differences in oxygen consumption in the two solutions

TABLE IV

SHOWING EFFECTS OF REDUCED OXYGEN TENSION UPON THE RATE OF OXYGEN CONSUMPTION IN *Daphnia*

APPROXIMATE SIZE OF BOTTLES—20 CC.

Normal Oxygen Tension, Control	Oxygen Tension at End of Experiment	5 Animals (Cubic Millimeters of Oxygen per Animal per Hour)
6.79	5.74	0.678
7.06	6.16	.547
7.41	6.16	.765
7.56	6.53	.774
7.62	6.65	.548
7.65	6.31	.690
7.64	6.52	.682
7.56	6.16	.864
6.98	5.48	0.913
6.98	5.12	1.123
Av. 7.33	6.08	0.758

APPROXIMATE SIZE OF BOTTLES—20 CC.

Reduced Oxygen Tension, Control	Oxygen Tension at End of Experiment	5 Animals (Cubic Millimeters of Oxygen per Animal per Hour)
4.39	3.28	0.750
5.67	4.86	.598
4.12	3.13	.679
3.02	2.50	.382
3.08	2.46	.414
3.49	2.36	.763
3.91	2.84	0.712
4.11	2.44	1.099
4.32	2.98	0.916
4.32	3.82	0.507
Av. 4.04	3.00	0.682

are tested statistically by Student's method, the figure of probability is 0.322, which is clearly not significant. This indicates that a difference in oxygen tension of 50 per cent of air saturation does not influence oxygen consumption in *Daphnia* sufficiently to be detected by the method used. However, when this series of experiments was repeated, using 8 cc. bottles with 2 animals in each, the average oxygen consumption in the air-saturated solution was 0.840 cu. mm. per animal per hour, as compared with 0.639 cu. mm. in the solution with oxygen tension reduced to 50 per cent of air saturation. The statistical probability in this case is 0.015, a significant value. Between these two sets of conflicting data the former should be given more weight because the experimental error in oxygen determinations was smaller in the larger volumes and larger numbers of animals. Since the differences in oxygen tension in these experiments are equal to or greater than the differences between groups and singles at the end of the experiments in Section 3 rather than at their beginning, it seems safe to assume that differences in oxygen tension have played at most only a minor part in causing differences in oxygen consumption in these experiments.

7. *The effects of carbon dioxide upon oxygen consumption in Daphnia.*—Three series of experiments were run, using 0.005 molar solution of potassium chloride as the toxic agent and 20 cc. bottles with ground-glass stoppers as containers. Each series of experiments consisted of two parts. The first part had 4 bottles with 20 animals in each in the KCl solution. The second part had 16 bottles with 2 animals in each in the same-strength solution which had previously had carbon dioxide bubbled slowly through it for 5 minutes. At the end of 6 hours oxygen determinations were made. Table V shows a summary of these results. In the bottles where 2 animals were present, the average oxygen consumption was 0.275 cu. mm. per animal per hour, as compared with 0.465 cu. mm. where 20 animals were present in the solution that had no carbon dioxide bubbled through it. When these results are treated statistically by Fisher's method of comparing means (Fisher, 1928, p. 107), the figure of probability is 0.0018, a quite significant value. These results indicate the importance of carbon dioxide as a retarding factor in metabolism of *Daphnia*.

8. *Effects of removing carbon dioxide from a 0.01 molar solution of calcium chloride upon the survival time of Daphnia living in it.*—

Watch glasses were used in these experiments instead of bottles so that more surface would be exposed. Ten cubic centimeters of 0.01 molar solution of calcium chloride were put in each watch glass. Five animals were put in each of 4 of the watch glasses, and single individuals in each of 20 of them. One-half of these, two groups of 5 and 10 singles, were sealed in a desiccator which had a strong solution of sodium hydroxide in its bottom chamber. The other half of the experiment was sealed in a desiccator without NaOH solution. Survival times of all *Daphnia* were carefully noted. Table VI shows

TABLE V

SHOWING EFFECTS OF CO_2 ON RATE OF OXYGEN CONSUMPTION IN *Daphnia*

Oxygen Tension in 0.005 Molar Solution KCl	20-Animal Groups (Mean Cubic Millimeters of Oxygen per Animal per Hour)	Oxygen Tension of 0.005 KCl Solution after CO_2 Was Bubbled Through	2 Animals, in CO_2 Bottles (Mean Cubic Millimeters of Oxygen per Animal per Hour)
8.0	0.482	6.65	0.240
8.2	.457	6.71	.261
8.0	0.457	6.61	0.323

TABLE VI

SHOWING EFFECTS OF REMOVING CO_2 SOLUTION UPON THE SURVIVAL TIME OF *Daphnia* LIVING IN IT

Exp. No.	Time in Hours	Number Surviving in 2 Groups of 5 Animals	NaOH, Number Surviving in 2 Groups of 5 Animals	Number Surviving in Singles, 10 Animals	NaOH, Number Surviving in Singles, 10 Animals
1	6	7	3	8	3
2	11	6	3	6	1
3	11	10	6	10	5
4	8	6	2	6	2
5	5	6	1	7	1
6	6	9	3	8	4
7	13	10	4	8	5
8	8	10	2	10	3
9	8	7	4	6	2
10	8	10	2	9	2
Total....	....	81	30	78	28

the results of these experiments. At the end of the experiment 81 of the group animals in the desiccator without NaOH solution were still surviving, as compared with 30 animals in the desiccator with NaOH solution. Likewise, 78 of the single animals were still surviving in the desiccator without NaOH solution, while only 28 singles were surviving in the desiccator with NaOH solution. When these results are treated statistically by Student's method, the following figures of probability are obtained:

	Probability
Groups in NaOH desiccator versus groups in other desiccator	0.0001 –
Singles in NaOH desiccator versus singles in other desiccator	0.0001 –

The minus signs after the probability figures indicate that the probabilities are less than those covered in Fisher's tables. These results show that the presence of carbon dioxide does influence the survival time of *Daphnia* living in a toxic solution.

All the evidence thus far indicates that the survival advantage of groups of *Daphnia* over that of single individuals when exposed to toxic solutions is due to slower rate of metabolism of the group, brought about largely by the accumulation of carbon dioxide in the limited volume of the group. This survival advantage of groups of *Daphnia* falls in line with Dr. Child's theory of differential susceptibility and agrees with his numerous demonstrations that various animals, when exposed to a large variety of injurious agents, survive longest in strong solutions when their rate of metabolism is slowest. The question naturally arises whether or not there is also differential acclimation between groups and single individuals in more dilute concentrations of toxic solutions where death does not occur too soon for some acclimation to take place.

In checking over all my available data, it was noted that the few times that singles lived longer than groups occurred in the more dilute concentrations of the toxic solutions. In order to test this point in more detail, a variety of chemicals were used and a series of survival experiments were made at different concentrations of each chemical solution to determine if there are concentrations in which single individuals will live longer than groups.

9. *Survival experiments with different concentrations of calcium-chloride solution.*—A series of 8 experiments were made with each of the following concentrations of molar solutions of calcium chloride: 0.025, 0.0375, 0.05, 0.065. Each experiment consisted of one group of 20 animals in 20 cc. of the solution in a half-ounce bottle, and 20 single animals one each in 20 cc. of the solution in a half-ounce bottle. Table VII shows the results of two concentrations: 0.0375 and 0.065. In the 0.065 solution there were 34 of the groups living at the end of the experiment and 3 of the singles. In the 0.0375 solution there were 57 of the singles living, as compared with 20 of the groups. In the 0.05 solution there were 36 of the singles surviving, while only 7 of the groups were living. In the 0.025 solution there were 103 of the singles living, as compared with 46 of the groups. When Student's method is applied to these data, the figures of probability are:

TABLE VII

SHOWING DIFFERENTIAL SURVIVAL BETWEEN GROUPS AND SINGLE *Daphnia* IN DIFFERENT CONCENTRATIONS OF $CaCl_2$ SOLUTION

0.0375 MOLAR SOLUTION $CaCl_2$ TIME, 10 HOURS		0.065 MOLAR SOLUTION $CaCl_2$ TIME, 7 HOURS	
Number Surviving in Singles	Number Surviving in Groups	Number Surviving in Groups	Number Surviving in Singles
6	1	4	1
4	1	3	0
5	1	7	0
7	2	5	1
7	2	5	0
7	2	2	0
9	5	3	1
12	6	5	0
57	20	34	3

	Probability
In 0.065 solution........	0.0001 group advantage
In 0.05 solution........	.016 single advantage
In 0.375 solution........	.0001 single advantage
In 0.025 solution........	0.001 single advantage

These figures of probability are all significant.

Table VIII shows a summary of the results of other chemicals treated similar to calcium chloride. In all these chemical solutions except sodium hydroxide and potassium hydroxide the groups survived better than singles in the more concentrated solution, and single individuals survived better than groups in more dilute solutions. In NaOH and KOH solutions the groups survived better than single individuals in all the concentrations tested. The results with sodium hydroxide and potassium hydroxide suggest that carbon dioxide produced by the animals reacts with the hydroxide solutions to form carbonates and water, thus making the hydroxide solutions more dilute; and since this process takes place more rapidly, other conditions being equal, the larger the numbers of animals present, the groups are able to survive longer than single individuals.

TABLE VIII

SHOWING SUMMARY OF OTHER CHEMICALS TREATED AS $CaCl_2$ IN TABLE VII

Chemical	Molar Concentration	Time in Hours	Probability, Group Advantage	Probability, Single Advantage
KCl	0.05	1.5	0.0008	
KCl	.025	6.75	....	0.002
$MgCl_2$	.05	18.0	.002	
$MgCl_2$	.037	43.0	....	.0001 –
$Mg(SO_4)_2$	.2	6.0	.0001	
$Mg(SO_4)_2$	.15	11.0	....	.005
$Ca(NO_3)_2$	.05	3.5	.0008	
$Ca(NO_3)_2$	.03	8.5	....	.0001 –
$Zn(NO_3)_2$	.015	3.5	.0001 –	
$Zn(NO_3)_2$	.001	15.0	....	.0008
$NaNO_3$	.135	1.2	.0001 –	
$NaNO_3$	.075	36.0	....	.0001 –
$NaCl$	.15	1.25	.0001 –	
$NaCl$	.05	66.0	....	0.0001 –
$NaOH$	.005	1.25	.002	
$NaOH$	.0006	28.0	.0001 –	
KOH	.0025	2.0	.0014	
KOH	0.00015	36.0	0.0001	

10. *Change in concentration of hydroxide solutions due to animals living in it.*—A large volume of dilute solution of sodium hydroxide was made in distilled water. From this stock solution 20 cc. were put in a number of half-ounce bottles. Some of these bottles were kept for controls without any animals in them. Two animals were put in each of 8 bottles, and 20 animals were put in each of 8 of

them. All were kept under identical conditions. After 3 hours the concentrations of these solutions were determined by titrating against hydrogen chloride with a known factor and using phenolphthalein and methyl orange as indicators. Table IX shows the results of these experiments. The mean molar concentration of the controls at the end of the experiments was 0.00094; this had been reduced to 0.000764 molar where 2 animals were present, and to 0.000606 molar when 20 were present. When the results of these experiments were expressed in percentages and treated statistically by Fisher's method for comparing means, the figure of probability is 0.0022. Identical experiments with dilute solution of potassium hydroxide gave the same type of results as in NaOH solution. The figure of probability in this case was 0.0001. Both of these figures of probability are quite significant, and the experiments show that groups of animals living in these solutions change the concentrations significantly over that of single individuals. These results give a satisfactory explanation for groups being able to survive longer than single individuals throughout the different concentrations of NaOH and KOH solutions.

TABLE IX

SHOWING CHANGES IN NaOH SOLUTION DUE TO *Daphnia* LIVING IN IT

Cubic Centimeters of NaOH Solution	Time in Hours	Molar Concentration		
		Control	2 Animals	20 Animals
20	3	0.000974	0.000575	0.000575
20	3	.000936	.000755	.000589
20	3	.000950	.000755	.000625
20	3	.000894	.000800	.000625
20	3	.000980	.000838	.000650
20	3	.000949	.000813	.000581
20	3	.000894	.000769	.000625
20	3	0.000940	0.000806	0.000581
Mean........	....	0.000940	0.000764	0.000606

11. *Effects of introducing carbon dioxide into a solution of calcium chloride upon the survival time of Daphnia living in it.*—The two concentrations of calcium chloride chosen for these experiments were the same as those which had been used in Section 9; at that time in

the 0.065 molar solution, groups survived longer than single individuals; in the other concentration, 0.025 molar solution, single individuals lived longer than the groups. Two sets of experiments were made with each of these concentrations. A large volume of 0.065 molar solution of calcium chloride was prepared. This was divided into two parts. From one part a set of experiments were made by putting 20 cc. of the solution into each of 88 half-ounce bottles. Twenty animals were put into each of 8 of these bottles, and 2 animals were put into each of 80 of them, making an initial number of 320 *Daphnia* equally divided between the two population densities. These were then paired off so that one group of 20 would be checked with 10 of the bottles containing 2 animals each. The other part of the solution had carbon dioxide bubbled slowly through it for 5 minutes; then an identical set of experiments were prepared as described above for the first part of the solution. These two sets of experiments were run simultaneously and under approximately the same environmental conditions. The survival times of all these were carefully checked. Table X shows the results of these experiments at definite times from which statistical calculations were made by applying Student's method.

Two sets of similar experiments were made with 0.025 molar solution of calcium chloride. Table XI shows the results obtained. When Student's method was applied to the results of the experiments with these two concentrations of calcium chloride, the following statistical probabilities were obtained:

At the end of 1 hour in the control 0.065 molar solution of calcium chloride, 76 of the groups were surviving, as compared with 4 of the single individuals; group advantage is indicated in this case with a probability figure of 0.0001. At the end of 2 hours in the 0.065 molar solution which had carbon dioxide bubbled through it, there were 62 of the grouped *Daphnia* surviving, as compared with 20 of the single individuals; the figure of the probability in this case is also 0.0001. At the end of 1.5 hours in the 0.065 molar solution, there were 10 animals of the groups living, as compared with 20 of the single individuals at the end of 2 hours in the 0.065 molar solution which had CO_2 bubbled through it; singles have a survival advantage in this case with a probability figure of 0.0016. At the end of 30 hours in the 0.025 molar solution, there were 132 single animals living, as compared with 70 animals of the groups; singles have a survival advantage in this case with a probability of 0.0075. At the end of 27 hours in the 0.025 molar solution which had CO_2, bubbled through it, 18 animals of the groups were surviving, as compared with 20 of the single animals; the probability figure in this case is 0.5006, which is not significant. At the end of 30 hours in the 0.025 molar solution without CO_2, there were 70 animals of the groups surviving, as compared with 18 single animals in the same solution, which had CO_2 bubbled through it, at the end of 27 hours.

TABLE X

SHOWING EFFECTS OF CO_2 UPON THE SURVIVAL TIME OF *Daphnia* IN 0.065 $CaCl_2$ SOLUTION

0.065 Molar Solution $CaCl_2$			
Time, 1 Hour		Time, 15 Hours	
Groups Surviving	Singles Surviving	Groups Surviving	Singles Surviving
10	2	2	0
10	1	2	0
10	0	1	0
10	0	1	0
8	0	0	0
10	1	3	0
9	0	0	0
9	0	1	0
76	4	10	0

CO_2 Bubbled for 5 Minutes through 0.065 $CaCl_2$ Solution			
Time, 2 Hours			
Groups Surviving	Singles Surviving	Groups Surviving	Singles Surviving
10	3	8	5
10	3	5	1
6	3	10	1
5	3		
8	1	62	20

An analysis of the foregoing data shows:

1. That the results of these experiments when CO_2 was not

bubbled through the solution agree with previous results of the same concentrations.

2. That bubbling CO_2 through 0.025 molar solution of calcium chloride destroys the normal survival advantage which singles have over groups at this concentration.

3. That bubbling CO_2 through 0.065 molar solution makes it possible for singles to survive longer than groups in the same concentration when no CO_2 has been bubbled through it, whereas groups normally live longer than singles at this concentration.

TABLE XI

SHOWING EFFECTS OF CO_2 UPON THE SURVIVAL TIME OF *Daphnia* IN 0.025 $CaCl_2$ SOLUTION

0.025 MOLAR SOLUTION $CaCl_2$		CO_2 BUBBLED 5 MINUTES THROUGH 0.025 MOLAR SOLUTION $CaCl_2$	
Time, 30 Hours		Time, 27 Hours	
Groups Surviving	Singles Surviving	Groups Surviving	Singles Surviving
10	18	2	1
5	18	2	3
12	15	3	3
12	15	2	3
4	18	1	3
10	15	4	3
15	15	3	2
2	18	1	2
70	132	18	20

4. That groups in 0.025 molar solution survive longer than singles in the same solution which has had CO_2 bubbled through it; whereas singles normally survive longer than groups at this concentration. In other words, the introduction of CO_2 into a solution where single individuals are living produces the same type of effect which is produced by groups of animals living in a small volume.

III. DISCUSSION AND CONCLUSIONS

There have been several theories postulated to explain the widespread phenomenon of differential survival of groups over that of single individuals when placed into a limited volume of injurious medium. These theories are briefly stated in the Introduction. Each theory has data to support it with the materials and under the conditions in which the experiments were made. It is reasonable to suppose that there is no one factor that will explain all this phenomenon of group protection as it occurs among phylogenetically widely differing groups of animals and under diverse conditions. It is probable that there are several factors working to produce group protection in each case where it has been observed, with perhaps one factor predominating in effect among one group of animals under certain conditions and a different factor predominating with a different group of animals under different conditions. The question which concerns us most here is: Which factor or factors have been the most influential in causing differential survival between groups and single individuals among *Daphnia* in these experiments?

Allee and Schuett (1927) showed that groups of *Planaria*, *Ophiderma*, and other aquatic animals lived longer than single individuals in colloidal silver because the groups produced slime in greater quantities than single individuals or otherwise adsorbed the colloidal silver and rendered the solution less toxic to animals comprising the group. Fowler (1927) showed that groups of *Daphnia* lived longer than single individuals in the same volume and concentration of toxic solution of numerous chemicals, chiefly electrolytes. In both cases the amount of chemical per animal would be much less in the group. The question as to whether groups of *Daphnia* living in a toxic solution weaken it more than would single individuals was tested by experiments recorded in Section 1 by the conductivity method of measuring concentrations of electrolytes. The results show that the concentration of an electrolyte, calcium chloride, was not changed significantly by the *Daphnia* living in it. If there are chemical reactions between the products of the *Daphnia* and their chemical medium, in so far as changes could be detected by the method used, the end-results of such reactions leave the medium with approximately the same degree of ionization.

For calcium chloride and presumably for other similar electrolytes, we have certainly not proved that with these animals chemical change of the medium is a major factor in group protection. On the other hand, in Section 10 data are given which demonstrate clearly that, when exposed to sodium or potassum hydroxide, *Daphnia* do alter the chemical composition of the solution, the more rapidly, other things being equal, the greater the number of animals present. Under the adverse conditions furnished by these alkalies, group protection is due to the removal of the toxic material as a result of the more rapid production of metabolic products (in this case carbon dioxide) by the group. Preliminary tests with the conductivity method did not detect the chemical changes which occur in the hydroxide solutions due to animals living in them, which means that carbonate ions were substituted for the hydroxyl ions in these solutions. However, this type of reaction does not seem to hold for the salts which were tested with the conductivity method in the foregoing experiments, because in the first place the affinity between the metals and chlorine to form salts is greater than that between the metal and carbon dioxide to form carbonates. Secondly, the fact that groups lived longer in strong concentrations of the salts than did single individuals, while single animals lived longer than groups in weaker concentrations, indicates that the effects of carbon dioxide were upon the animals instead of reacting with the chemical medium, as in the cases with hydroxides, where the groups survived longer than single individuals throughout all the concentrations tested.

The results of the experiments with tubes having bottoms of cheesecloth (Section 2) where the groups and single individuals were kept in the same solution but limited in their environment indicate that there is an influence of some kind which gives advantage to the groups. These results show also that this influence can be eliminated almost, if not entirely, by frequently lifting the tubes out of the solution and thoroughly mixing the medium. These experiments do not make clear what this influence is. It may be the result of contact among members of the group, which Bilski and Goetsch showed leave an adverse effect upon survival or which Allee (1927) and Drzewina and Bohn (1928) report has positive survival value under certain conditions. It may also be the result of secretions (Drzewina and Bohn, 1921); or it may be that excretory products of the group or the lessened oxygen tension brought about by the group influences their metabolic rate and hence causes differential survival.

Further studies of the influence of grouping animals together are reported in Section 3, in which oxygen consumption is measured. These results show a marked reduction in the rate of oxygen consumption of the group as compared with singles. This means that metabolism has been greatly reduced in the individuals composing the groups, due probably to one or more of the factors given in the preceding paragraph. Experiments with aërated conditioned solution and raw solution of calcium chloride (Section 4, Table III) throw some light on the subject. Since single individuals living in unaërated conditioned solution consume oxygen no faster than the groups in raw solution of the same concentration, it is evident that the causes for retarding metabolism in groups are retained in the solution after the animals are removed.

Experiments with aërated conditioned solution and raw solutions of calcium chloride (Section 5) throw further light on the subject. Since single individuals living in aërated conditioned solutions do not have their metabolism retarded and consume oxygen much faster than groups in raw solution of the same concentration, it is evident that factors retarding metabolism among groups of *Daphnia* are of a volatile nature and are eliminated from a solution by aërating it or result from low oxygen tension. This suggests that the metabolic rate of groups is retarded either by accumulation of carbon dioxide which is given off into the medium in greater quantities by the group or by reduced oxygen tension in the medium of the group. The results of experiments upon the effects of reducing oxygen tension in calcium chloride solution to about 50 per cent of air saturation upon the rate of oxygen consumption of animals living in it tend to minimize the importance of this factor in these experiments (Section 6, Table IV). The larger numbers of animals in the larger-sized bottles show no significant difference in oxygen consumption between animals living in a solution of air saturation and animals living in the same concentration of calcium chloride with the initial oxygen tension at 50 per cent of air saturation. However, the results obtained from an analysis based on the exposure of smaller number of animals in smaller bottles show exactly the opposite results. The former set of data should be given more weight on account of experimental error being considerably less with the larger numbers of animals in larger volumes of solution. This conclusion that oxygen tension is not an important factor in group protection is further strengthened by the fact that the usual survival experiments were run in uncorked wide-mouth bottles which allowed continuous aëration. Still, the results in the latter case must be considered as indicative that reduced oxygen tension may play a rôle in retarding metabolism among groups of *Daphnia* in a limited volume which is not exposed to the air. An examination of the data in the foregoing experiments indicates that it is very improbable that differences in oxygen tension even in closed vessels have been an important factor in reducing metabolism, because the rate of metabolism was reduced relatively more in the groups of intermediate size (Section 3) than in the largest groups, where oxygen tension was most reduced. When oxygen consumption of these intermediate-sized groups is compared with that of single individuals in the same concentration of calcium chloride, the rate is significantly lower among the groups even when the oxygen tension is 85 per cent of air saturation at the end of the experiment. On the other hand, single individuals (Section 5) introduced into an aërated conditioned solution of calcium chloride with an initial oxygen tension at 80 per cent of air saturation did not have their rate of oxygen consumption appreciably lowered as compared with that shown by groups of animals in raw $CaCl_2$ solution of the same concentration with oxygen tension at air saturation. Since groups of intermediate numbers have their rate of metabolism significantly lowered when oxygen tension in 85 per cent of air saturation at the end of the experiment, while single individuals do not have their rate of metabolism appreciably lowered even when oxygen tension is 80 per cent of air saturation at the beginning of the experiment, it seems safe to assume that differences in oxygen tension have played, at most, only a minor rôle in reducing the rate of metabolism among groups of animals in these experiments. These conclusions agree, on the whole, with the results of many recent investigations which show that the rate of oxygen consumption among animals is independent of oxygen tension down to about 50 per cent or less of air saturation (Lund, 1921; Garrett, 1928; Helff, 1929).

The other factor indicated as possibly influential in reducing the rate of metabolism of groups of *Daphnia* is the accumulation of carbon dioxide in their medium, which is produced in larger quantities by groups of animals than by single individuals. It is interesting to note that introducing carbon dioxide into a solution of potassium chloride (Section 6, Table V) markedly lowers the rate of metabolism of animals living in it. Even the rate of oxygen consumption of single individuals is reduced to about one-half that of groups living in the same concentration of potassium chloride when no carbon dioxide has been added; whereas groups in previous experiments consumed only about one-half as much oxygen as single individuals. It is evident here, also, that oxygen tension plays little or no part in reducing the rate of metabolism, because the groups in these experiments have the same reduced oxygen tension as do others discussed in Section 3, where single individuals consumed oxygen much faster than groups. These results give us ample evidence that carbon dioxide is a very important factor in reducing the rate of metabolism in grouped *Daphnia*, and it is very probable that accumulation of carbon dioxide has been the most important factor in reducing the rate of metabolism of groups in all these experiments.

When carbon dioxide is removed from a solution in which animals are living (Section 8, Table VI), their survival times are greatly reduced. It appears again from these results that accumulation of carbon dioxide among groups of *Daphnia* may be a very important factor in causing group protection by slowing down their rate of metabolism and thus, through differential susceptibility, allowing groups to survive longer than single individuals (Child, 1915, 1924). This makes it possible to explain group protection in *Daphnia* exposed to relatively highly toxic solution in terms of Child's theory of differential susceptibility. If this is true, then according to the other part of this theory there should be differential acclimation in the weaker concentrations of toxic solutions. Under such conditions single individuals, which have a higher rate of metabolism than do those in groups, should survive longer than groups, thus giving the type of results which Drzewina and Bohn (1921–22) ascribe to an autodestructive secretion in the cases of *Polycelis* and *Convoluta* in KCl solutions.

Experiments reported in Section 9, Tables VII and VIII, give considerable data on this subject covering a number of different chemical solutions. These data demonstrate differential susceptibility and differential acclimation between groups and single individuals in all cases except in NaOH and KOH solutions. In these two solutions there is differential susceptibility only, that is, groups survived longer than singles in all cases tested in these solutions. The experiments with NaOH and KOH solutions (Section 10, Table IX) furnish data to show that carbon dioxide reacts with the solutions to form carbonates and water, thus weakening the toxic concentration of the medium and permitting the groups to survive longer than the single individuals. Under these conditions differential acclimation would not take place.

Further evidence that CO_2 is an important factor in producing differential susceptibility and differential acclimation is obtained by introducing carbon dioxide into a strong solution of calcium chloride where single animals are living. One can increase the survival time of the singles until they live significantly longer than groups in the same concentration of calcium chloride. In more dilute solutions of calcium chloride, where singles ordinarily survive longer than groups, one can use the same method and decrease the survival time of the singles until the groups survive significantly longer (Section 11, Tables X and XI). In other words, by varying the amount of carbon dioxide in a solution of calcium chloride, it is possible to reverse differential susceptibility and differential acclimation between groups of *Daphnia* and single individuals.

After considering all the data at hand, it seems safe to conclude that the differential rate of accumulation of carbon dioxide in the medium is the most important factor in influencing differential survival between groups of *Daphnia* and single individuals in these experiments. These results do not disprove other theories of group protection with other animals or even with *Daphnia* under different conditions. They do demonstrate a hitherto neglected factor which must be considered when survival time between groups of animals and single individuals is being studied.

This type of differential survival between groups and single individuals may be of considerable biological importance to *Daphnia*, furnishing, as it does, two survival mechanisms, one of which is suitable for a sparse population in a mildly injurious environment, while the other acts when many *Daphnia* are crowded together in the presence of more extreme toxicity. Under these conditions some members of the race have a chance of survival if the toxicity be not too great or of too long duration.

IV. SUMMARY

1. *Daphnia* living in a $CaCl_2$ solution do not alter its electrical conductivity to any appreciable extent.

2. Numbers of *Daphnia* living in a limited volume of $CaCl_2$ solution consume significantly less oxygen than single individuals living in the same concentration of this electrolyte.

3. Single individuals living in unaërated conditioned solutions of calcium chloride have their rate of oxygen consumption lowered to that of groups in the same concentration of raw solution.

4. Single individuals living in aërated conditioned solutions of calcium chloride do not have their rate of metabolism lowered to any appreciable extent.

5. Reduced oxygen tension has very little influence on oxygen consumption in *Daphnia*, at least down to 50 per cent of air saturation.

6. Diffusion of carbon dioxide into a solution markedly lowers the rate of oxygen consumption of *Daphnia* living in that solution.

7. Removal of carbon dioxide from a solution of calcium chloride significantly shortens the survival time of *Daphnia* living in it.

8. Groups of *Daphnia* survive longer than single individuals in stronger concentrations of a variety of toxic solutions, while single individuals survive longer than groups in weaker concentrations of the same solutions.

9. *Daphnia* living in dilute solutions of sodium hydroxide and potassium hydroxide significantly weaken the concentration of these chemicals.

10. Introducing carbon dioxide into toxic solutions where *Daphnia* are living increases their survival time in strong concentrations and decreases their survival time in weak concentrations.

11. Differential survival between groups of *Daphnia* and single individuals can be explained on the basis of Child's theory of differential susceptibility and differential acclimation of slowly and rapidly metabolizing animals.

12. All these data indicate that carbon dioxide is a very important factor in causing differential survival between groups of *Daphnia* and single individuals when exposed to toxic solutions.

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THE INFLUENCE OF VARYING TENSIONS OF OXYGEN UPON THE RESPIRATORY METABOLISM OF CERTAIN AQUATIC INSECTS AND THE CRAYFISH

(Four figures)

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PHYSIOLOGICALLY insects differ from the vertebrates in many ways. The respiratory system, for example, of the arthropods is unique in the animal kingdom. Blood can be considered the chief respiratory organ in the vertebrates, while the other organs of respiration are mainly accessory to it. Among the insects the tracheal system has received most of the credit, the body fluids having been considered of little importance. In recent years, however, insect blood has come to be recognized as playing a more and more important rôle in respiration. Little at present is understood about the respiratory pigments of insect blood. Muttkowski (1921) has demonstrated the presence of copper in the blood of insects in amounts proportional to that found in hemocyanin. Among the highly organized active insects the need of such respiratory aids seems imperative. It appears impossible for oxygen to reach the tissues rapidly enough by simple gaseous diffusion through the tracheal walls and body fluids to supply the needs of the body. Of course, the oxygen demand of poikilothermal animals is not as great as that of warm-blooded forms, since the needs of the latter include oxygen for body heat as well as for endogenous metabolism.

To serve as a comparison with the insects, the crayfish was selected as an organism known to have the respiratory pigment, hemocyanin, present in the blood plasma, and also effective ventilating and circulating mechanisms. The aquatic insects employed are not truly tracheate forms, but rather apneustic ones which take their oxygen from the water in dissolved form. Hence, if respiratory aids are to be looked for among the insects, one should expect to find them among the water-breathers.

Two types of relationship may exist between the oxygen consumption of the animal and its environment. First, the organism may have some regulatory apparatus by means of which its oxygen consumption may be kept constant over a wide range of oxygen tensions. Such individuals are found among the vertebrates almost without exception, and to a considerable extent among the invertebrates. The Protozoa and bacteria also belong to this group. A slightly different aspect is presented by the unicellular forms. These have no need of a regulating mechanism, since their absorptive surface is proportionately much greater than that of the Metazoa, due to their small size. Among this first type of relationship we should expect to find organisms amply supplied with respiratory aids such as pigments and ventilating systems. Secondly, the organism may lack a mechanism for regulatory control so that its oxygen consumption is dependent upon the oxygen tension of the medium. In this type of relationship we should expect to find less active forms, and ones which live under more uniform conditions. There are varying degrees of dependence of the organism's respiration upon oxygen tension. Some forms are dependent upon the entire range of tensions to such an extent that the oxygen intake is at all times directly proportional to the oxygen tension of the surrounding medium. Examples of this type are found among the echinoderms (Hyman, 1929). Others may be less dependent over part of the range, i.e., may possess some ability to regulate their respiration, at the same time not being able to do so completely.

The purpose of the following experiments was to determine to what extent the insects and the crayfish were independent of oxygen tensions of the water. As will be shown, the degree of independence depends not only upon the tension of oxygen but also upon the rate at which the tensions diminish.

HISTORICAL

Doctor Hyman, in her paper appearing in a preceding number of this journal, has given an excellent summary of the investigations to date. To this might be added the work of the following investigators. Shoup (1929) found the oxygen consumption of luminous bacteria to be fairly constant over a wide range of oxygen tensions down to about 9 mm., below which it drops off more abruptly. With fishes, additional work has been done by Wells (1913), Powers and Shipe (1928), Hall (1929), and Keys (1930). Keys has shown that the respiration of *Fundulus* is relatively independent of small deviations from the usual oxygen tension of the medium, and also that the respiration of fishes is very markedly decreased at or near the level of asphyxia. Hall, using three species of marine fish—the scup, puffer, and toadfish—reports a correlation between the activity of the fish and the influence of oxygen tension, such that the more active fishes show a greater resistance to variations in oxygen tension. He also correlated the degree of respiratory independence with the amount of hemoglobin present per unit volume of blood. In other words, the form with the lowest percentage of hemoglobin (the toadfish) was the most dependent upon the oxygen tension of the surrounding water. Powers and Shipe report a lowered rate of oxygen absorption in the herring, silver salmon, and viviparous perch, as the oxygen tension of the sea water is decreased. They also showed the effects of carbon dioxide upon the oxygen consumption of the above fishes. The work of Hall and of Powers and Shipe has been criticized by Keys. Wells demonstrated that fishes are much more sensitive to changes in the carbon dioxide than in the oxygen content of the surrounding water.

As far as the author is aware, only two investigators have reported any results with insects. Both used the same form, the meal worm, *Tenebrio*, but in different stages of metamorphosis. Gaarder (1918) has shown that the pupae of *Tenebrio* respire at a constant rate in oxygen tensions from 5 to 97 per cent. Thunberg (1905) has demonstrated that meal-worm larvae show a marked decrease in oxygen consumption in atmospheres of 10 per cent oxygen or less. No results are reported for aquatic insects.

MATERIAL

Two species of aquatic insects were used. One form, the nymph of the dragon fly, *Nasiaeschna penthacantha*,[1] represents a water-breathing insect which has the ability to ventilate actively its absorptive surface by drawing water into and out of the rectum.

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[1] Donated by J. H. Roberts, Baton Rouge, Louisiana.

This type of ventilation can be compared favorably to the passage of water over the gills of fishes. The other apneustic form was the larva of the caddis fly, *Limnephilus rhombicus*. Respiration in this insect takes place through small filamentous gills and through the body wall. It is perhaps not as effective a system as that of the dragon-fly nymph, inasmuch as the body is covered by a protective case which to some extent hinders the circulation of water. However, the caddis worm is a smaller insect with relatively larger body surface for oxygen absorption. Furthermore, it manages to maintain a slight circulation of water through the case by movements of the body, as can be demonstrated by placing carmine in the water at the mouth of the tube. Caddis-fly larvae were obtained from one of the small streams emptying into the south shore of Lake Wingra in the vicinity of Madison, Wisconsin.

The crayfish, *Cambarus virilis*, was used to serve as a comparison with the insects. Individuals were obtained from under stones in the Yahara River, also in the vicinity of Madison. Only small crayfish were used, two being employed at a time, with a total weight not in excess of 15.2 gm. When placed in the wire-screen cage suspended in water, the crayfish successfully kept the water stirred by their scaphognathites, so that no special stirring apparatus was necessary. As all forms were suspended in the middle of the jar, there was no appreciable stratification of gases.

APPARATUS AND METHODS

Oxygen was measured by the micro-Winkler of Lund (1921). Carbon dioxide was determined by Seyler's method as described by Sutton (1924). By measuring the amount of carbon dioxide present in the water before and after each experiment, its effect upon respiration could be shown. Carbon-dioxide figures are not to be considered exact since the end-point is difficult to obtain. The procedure was as follows.

The organisms were placed in a wire-screen cage having a mesh of about 2.0 mm. This cage was cylindrical, with circular caps for each end. The caps were held in place by a gum rubber band, and the entire cage suspended by a silk thread from a hook in the stopper of the bottle (see Fig. 1). The jar, when stoppered, had a capacity of

960 cc. Into the side of the jar was cemented a capillary stopcock to allow samples of water to be withdrawn at will. Through the stopper were inserted a thermometer and a bent capillary tube extending to the bottom of the jar. Outside the jar a 50-cc. burette with a screw cock was attached to the bent capillary tube. The burette was filled with clean mercury, which was allowed to run into the jar whenever samples of water for oxygen analysis were drawn through the glass stopcock. If a circulation of water was desired, it was necessary only to rock the jar occasionally. As the wire-screen cage was suspended by a thread, it acted as a paddle. However, the results were no different whether the jar was agitated in this manner or not. In all tests, unless otherwise stated, distilled water was employed so that the carbon dioxide produced could be measured as free, and not bound in the form of carbonates and bicarbonates. The hydrogen-ion concentration was determined by the colorimetric method using prepared buffer solutions of known pH. Samples for oxygen determination were drawn off into 10-cc., glass-stoppered, volumetric flasks. At each sampling, 12 cc. of mercury were admitted from the burette to allow the surface water to overflow the flask. Determinations were then carried out on 10 cc. of the water. The reagents were admitted to the bottom of the volumetric flasks by means of slender pipettes drawn out to a fine point and calibrated to deliver 0.1 cc. Titrations were carried out with N/4000 sodium thiosulphate in white porcelain evaporating dishes. Using a 10-cc. sample, each cubic centimeter of thiosulphate is equivalent to 2.0 mg. of oxygen per liter, or 1.399 cc. of oxygen at 0° and 760 mm. The thiosulphate was checked frequently with a standard potassium dichromate solution.

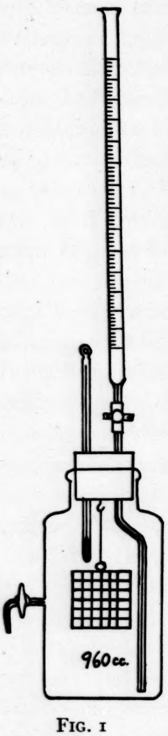

FIG. 1

Adequate tests were carried out to determine if mercury or distilled water had any injurious effects upon the animals. It was found that the organisms were not influenced to any appreciable effect by either.

A slight variation was made in the cage used to house the crayfish. It was made about twice as long as wide to allow the animals to remain in a normal, uncramped, horizontal position.

EXPERIMENTS WITH NYMPHS OF THE DRAGON FLY (*Nasiaeschna penthacantha*)

The nymphs were starved for 4 days before the experiments were performed, in order to allow time for their metabolic rate to become normal. During all the experiments they behaved admirably, scarcely moving about until toward the end of the tests, when the oxygen consumption had practically ceased. It was clearly evident when the oxygen tension had been reduced to a point "uncomfortable" for the insects. At the end of each experiment the nymphs were removed to running water.

Experiment I. To determine the normal effect of diminishing oxygen tensions upon the rate of oxygen consumption.—Seven individuals with a total weight of 4.227 gr. were used. They were placed in a cage suspended in distilled water with a pH of 6.35 and were allowed to reduce the oxygen tension by their own respiration. Samples were withdrawn every 30 minutes and analyzed for oxygen content. Carbon dioxide was determined at the start and end of the experiment. It was decided at the conclusion of the first experiment that a better average could be had if samples were withdrawn every 60 minutes instead of 30. In this way less water would be removed from the total amount, and yet a sufficient number of points would be had for plotting the results. The results of the first experiment are shown in Table I and Fig. 2 (I). If the amount of oxygen (milligrams per liter) is plotted against time (diminishing oxygen tension), it can be seen that from 7.36 to 3.96 mg. of oxygen per liter the oxygen consumption is fairly uniform, but that below 3.96 per liter, consumption grows less as the tension diminishes. In other words, oxygen consumption is fairly uniform down to about one-half the normal saturation of water. Following this first experiment, one of the nymphs was found dead, but the rest were apparently normal. The remaining six were weighed and used throughout the tests with no other casualties. Since the weight was reduced by the death of one of the nymphs, it became necessary to reweigh the remaining ones and to take a second normal test.

Experiment II. To determine the normal effect of diminishing oxygen tensions upon the rate of oxygen consumption (as in Experiment I).—Six individuals, with a total weight of 3.637 gr., were used. Water of the same pH as Experiment I, and of approximately the same carbon-dioxide content, was used. Samples from here on were taken at intervals of 60 minutes except as the end of the experiment was reached.

TABLE I

Nasiaeschna penthacantha: EXPERIMENT I

7 specimens. Wt., 4.227 gr. pH of water, 6.35.
Temp., 23° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.68	7.36	4.73
30	948	3.50	7.00	
60	936	3.34	6.68	
90	924	3.06	6.12	
120	912	2.95	5.90	
150	900	2.80	5.60	
180	888	2.72	5.44	
210	876	2.50	5.00	
240	864	2.30	4.60	
270	852	2.10	4.20	
300	840	1.98	3.96	
330	828	1.90	3.80	
360	816	1.85	3.70	
390	804	1.80	3.60	9.29

The results of the second experiment are also shown graphically in Figure 2 (II). The similarity of this curve with that obtained in the previous experiment with seven animals can be seen. The six nymphs required a longer time to lower the tension, both because of their decrease in number and because samples of water were withdrawn at longer intervals. It can be questioned at this point whether the decrease in oxygen consumption toward the end of the test was not due to the accumulation of carbon dioxide produced by the organisms, and not simply to the lowering of the oxygen tension. To determine the effects of carbon dioxide on the curve of oxygen consumption the following experiment was performed.

Experiment III. To determine the effect of diminishing oxygen tensions upon the rate of oxygen consumption in the presence of relatively high carbon-dioxide content.—The same six individuals were again used. Using distilled water as before, carbon dioxide generated by the action of very weak HCl upon $CaCO_3$ was bubbled through it for several minutes. The bottle containing the water was then stoppered and shaken to thoroughly dissolve the gas. Water was added to fill the bottle to the cork, which was pushed in firmly to prevent escape of carbon dioxide. The following morning the experiment was started, using this water with a carbon-dioxide content of 92.4 mg. per liter. The pH of the water was lowered by the H_2CO_3 thus formed from 6.35 to 5.2.

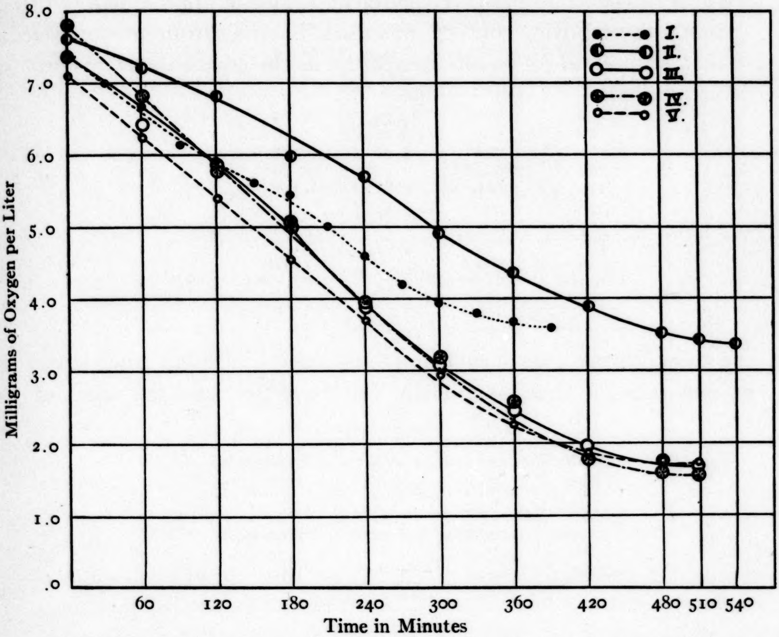

FIG. 2.—*Nasiaeschna penthacantha:* Oxygen consumption in relation to oxygen tension. Curves are numbered to correspond to their respective experiments.

Graph III (Fig. 2) shows the type of respiration curve obtained.

Two differences are apparent. First, the rate of oxygen consumption in the high carbon-dioxide tension was greater than normal. Second, the animals were able to continue to consume oxygen to much lower oxygen tensions than previously. One might explain the effect of carbon dioxide as stimulating the respiration of the insects so that their rate of oxygen consumption was increased and remained so for a longer period than normal. There is the possibility here that a lowering of the pH of the medium, and not the carbon dioxide pres-

TABLE II

Nasiaeschna penthacantha: Experiment II

6 specimens. Wt., 3.637 gr. pH of water, 6.35.
Temp. 23° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.80	7.60	4.18
60	948	3.60	7.20	
120	936	3.40	6.80	
180	924	2.98	5.96	
240	912	2.85	5.70	
300	900	2.45	4.90	
360	888	2.20	4.40	
420	876	1.95	3.90	
480	864	1.76	3.52	
510	852	1.72	3.44	
540	840	1.70	3.40	7.70

TABLE III

Nasiaeschna penthacantha: Experiment III

6 specimens. Wt., 3.637 gr. pH of water, 5.2.
Temp. 23° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.68	7.36	92.4
60	948	3.20	6.40	
120	936	2.94	5.88	
180	924	2.50	5.00	
240	912	1.95	3.90	
300	900	1.55	3.10	
360	888	1.24	2.48	
420	876	0.98	1.96	
480	864	0.90	1.80	
510	852	0.86	1.72	95.7

ent, caused the change. With this in mind, Experiment IV was conducted to determine the effect of a lowering of the pH in the absence of carbon dioxide.

Experiment IV. To determine the effect of lowering the pH of the water upon the rate of oxygen consumption in diminishing oxygen tensions.—Distilled water from the same source as that used in the previous experiments was used. Weak HCl was added drop by drop until the pH was lowered from 6.35 to 4.35. The carbon-dioxide content of the water was measured before the acid was added, and was found to be 4.84 mg. per liter, or about the same as that of Experiment II. The results of this test (Graph IV, Fig. 2) are very similar to those of the preceding trial. The effect of a low pH (with low carbon-dioxide content) is apparently the same as that of a fairly high carbon-dioxide tension. The question then arose as to what would be the effect of a combination of these two factors.

TABLE IV

Nasiaeschna penthacantha: EXPERIMENT IV

6 specimens. Wt., 3.637 gr. pH of water, 4.35.
Temp., 23° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.90	7.80	4.84
60	948	3.40	6.80	
120	936	2.94	5.88	
180	924	2.54	5.08	
240	912	1.96	3.92	
300	900	1.60	3.20	
360	888	1.30	2.60	
420	876	1.15	2.30	
480	864	1.05	2.10	
540	852	1.04	2.08	8.80

Experiment V. To determine the combined effects of a low pH and high carbon-dioxide content (in carbonate-free water) upon the rate of oxygen consumption in diminishing oxygen tensions.—In this experiment carbon dioxide was again bubbled through the water for a longer time until a great amount of it was absorbed. Consequently the pH was lowered from 6.35 to 4.2. Thus both pH and carbon-dioxide content were changed. Upon determination of carbon dioxide it was found that 225.5 mg. were present per liter. The results of this trial are shown in Fig. 2 (Graph V). It can be seen that no increase in the rate of oxygen consumption over that of trials III and IV resulted. In other words, the maximum effect was obtained by either of these factors alone, and no augmentation occurred when both were used together.

TABLE V

Nasiaeschna penthacantha: EXPERIMENT V

6 specimens. Wt., 3.637 gr. pH of water, 4.2.
Temp., 23° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.55	7.10	225.5
60	948	3.16	6.32	
120	936	2.70	5.40	
180	924	2.28	4.56	
240	912	1.90	3.80	
300	900	1.48	2.96	
360	888	1.15	2.30	
420	876	0.95	1.90	
480	864	0.90	1.80	
510	852	0.87	1.74	225.5+

EXPERIMENTS WITH CADDIS-FLY LARVAE
(*Limnephilus rhombicus*)

Caddis-fly larvae were chosen as a type of apneustic insect which lacks a well-developed circulating system. It was thought by the writer that perhaps these forms would show a greater dependence upon oxygen tension than the others. The experiments were carried on by the same method as that of the dragon-fly nymphs. Groups of 15 and 25 larvae were used. No weights are given, as it would be impossible to weigh the insects without first removing them from their cases, a procedure which usually results disastrously to the larvae when tried. However, it is not necessary to know the weight since they were used in large enough numbers to form a fair average and since, as will be shown, the respiration of two groups of the same number was practically the same. Regardless of the size of their cases, the worms were of approximately the same age and nearly the same size.

Experiment I. To determine the normal relationship of oxygen consumption to the oxygen tension of the water.—In this experiment 15 larvae were selected of the same species from the same location in the stream. The cases were approximately alike in size and structure. They were placed in distilled water with a pH of 6.3 containing 33.0 mg. of carbon dioxide per liter. Samples for the determination of oxygen were withdrawn each hour until it was evident that the oxygen consumption had practically ceased. The results are shown in Table VI and Graph I (Fig. 3). A straight-line relationship is shown to practically the level where oxygen consumption ceases, thus indicating an ability to regulate respiration down to a very low level of oxygen tension.

TABLE VI

Limnephilus rhombicus: EXPERIMENT I

15 individuals. pH of water, 6.3. Temp., 23.5° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.60	7.20	33.0
60	948	3.22	6.44	
120	936	2.95	5.90	
180	924	2.82	5.64	
240	912	2.42	4.84	
300	900	2.23	4.46	
360	888	1.80	3.60	
420	876	1.60	3.20	
480	864	1.20	2.40	
540	852	0.98	1.96	
600	840	0.95	1.90	
630	828	0.82	1.64	
690	816	0.80	1.60	37.4

Experiment II. To determine the effect of high oxygen tensions upon the rate of oxygen consumption.—Twenty-five caddis worms were used in distilled water with a pH of 6.6. The oxygen tension was raised by shaking water in a bottle containing pure oxygen. The water was then poured into a smaller bottle and stoppered with no air space above the water. In this manner water with an oxygen tension about twice normal was obtained. A larger number of insects (25) was used in order to produce a more pronounced effect. The experiment was not carried on until oxygen consumption had ceased, but was run for 8 hours, at the end of which time the oxygen tension was reduced to about normal equilibrium of air and water. Thus the effects of oxygen tensions above normal were demonstrated.

The results of this trial (Graph II, Fig. 3) show that the oxygen consumption was practically uniform throughout all tensions of 15.16–6.76 mg. of oxygen per liter. Thus, an increase in oxygen concentration above normal in no way alters the normal respiration rate. Following this experiment the same 25 individuals were placed in distilled water of normal oxygen tension which was slightly higher than that of the end of Experiment II.

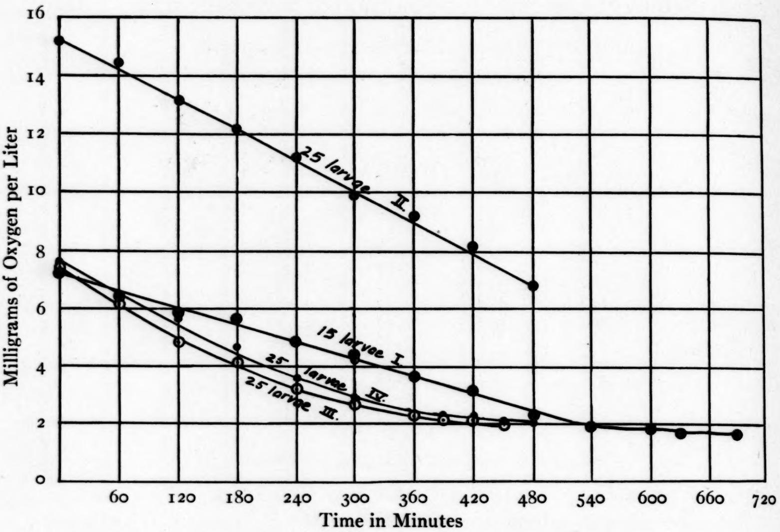

FIG. 3.—*Limnephilus rhombicus:* Oxygen consumption in relation to oxygen tension. Curves are numbered to correspond to their respective experiments.

Experiment III. To determine the effect of oxygen tensions below normal upon the rate of oxygen consumption of the same set of individuals used in the preceding experiment.—On the following day the same insects were placed in water with a pH of 6.8 having an oxygen tension of 7.48 mg. per liter. When plotted (Graph III, Fig. 3), the points obtained in Experiment III lie on a curve. However, assuming experimental error, the first four or five points might possibly represent a straight-line relationship as well as a curve. A distinct curve is shown at tensions below 3.20 mg. of oxygen per liter. The difference between Graphs I and III is evidently due to the number of insects used, a point which will be taken up later in the discussion.

TABLE VII

Limnephilus rhombicus: Experiment II

25 individuals. pH of water, 6.6. Temp., 22.5° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	7.58	15.16	29.7
60	948	7.23	14.46	
120	936	6.62	13.24	
180	924	6.10	12.20	
240	912	5.60	11.20	
300	900	4.98	9.96	
360	888	4.60	9.20	
420	876	4.08	8.16	
480	864	3.38	6.76	37.4

TABLE VIII

Limnephilus rhombicus: Experiment III

Same 25 individuals as in Experiment II.
pH of water, 6.8. Temp., 22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.74	7.48	6.60
60	948	3.10	6.20	
120	936	2.40	4.80	
180	924	2.08	4.16	
240	912	1.60	3.20	
300	900	1.38	2.76	
360	888	1.12	2.24	
390	876	1.08	2.16	
420	864	1.02	2.04	
450	852	0.98	1.96	14.30

Experiment IV. A repetition of Experiment III.—The same 25 animals were used again in this experiment to determine the similarity of curves obtained with the same organisms under practically the same conditions at a different time. The results of Experiment IV are shown by Graph IV, Figure 3. The oxygen tension of the water was slightly higher at the start than in Experiment III, but the two curves appear to be very similar in general appearance. In fact, the slight differences cannot be considered of any consequence.

TABLE IX

Limnephilus rhombicus: EXPERIMENT IV

Same 25 individuals. pH of water, 6.78. Temp., 22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.80	7.60	8.80
60	948	3.24	6.48	
120	936	2.88	5.76	
180	924	2.32	4.64	
240	912	1.80	3.60	
300	900	1.45	2.90	
360	888	1.15	2.30	
420	876	1.12	2.24	
450	864	1.06	2.12	
480	852	1.00	2.00	18.70

TABLE X

Limnephilus rhombicus: EXPERIMENT V

Second set of 25 individuals. pH of water, 6.78.
Temp., 22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)	Carbon Dioxide per Liter (Mg.)
0	960	3.95	7.90	6.6
60	948	3.30	6.60	
120	936	2.60	5.20	
180	924	2.15	4.30	
240	912	1.65	3.30	
300	900	1.50	3.00	
360	888	1.24	2.48	
420	876	1.05	2.10	
450	864	0.98	1.96	
480	852	0.98	1.96	17.6

Experiment V. To determine the similarity of respiratory curve, using a second set of the same number of animals under the same conditions as in Experiments III and IV. Twenty-five other caddis-fly larvae were tried in order to serve as checks with Experiments III and IV. The results of this trial are not shown in Figure 3 for the sake of clarity. However, the cure thus formed is practically identical with Graph IV, showing that the respiration of the second group of 25 animals was practically the same as that of the first.

EXPERIMENTS WITH THE CRAYFISH (*Cambarus virilis*)

Instead of distilled water, city tap water with a pH of 7.8 was used in all experiments. This was very "hard" water, being well buffered with alkali carbonates. The determinations of carbon dioxide in such waters, in which it exists both as bound and free, were not reliable; so they are not recorded. The same stock water was used in each experiment, assuming it to be uniform in carbonates in each case. The purpose of the experiments with the crayfish was primarily to determine the degree of dependence upon oxygen tensions; however, some interesting results other than this were obtained which will no doubt aid in accounting for the differences in results of previous workers.

TABLE XI

Cambarus virilis: EXPERIMENT I

2 individuals. Total wt., 11.867 gr. pH of water, 7.8.
Temp., 19.5°–20.5° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)
0	960	4.58	9.16
60	948	4.05	8.10
120	936	3.52	7.04
180	924	3.00	6.00
240	912	2.22	4.44
300	900	1.86	3.72
360	888	1.42	2.84
420	876	1.30	2.60
450	864	1.15	2.30
480	852	1.12	2.24

Experiment I. To determine the normal effect of diminishing oxygen tension upon the rate of oxygen consumption.—Two small individuals with a total weight of 11.867 gm. were used. They were placed in the wire cage described under "Materials and Methods," and allowed to reduce the oxygen tension by their own respiration. The results are shown in Figure 4, Graph I. Oxygen consumption is practically constant from normal tension to that of 2.84 mg. per liter. Below the latter, oxygen consumption drops off as the tension decreases, until a point as low as 2.24 mg. is reached. This would indicate that these two individuals were independent of most of the range and could regulate their respiration until quite a low point was reached. In the following experiment two other crayfish were used.

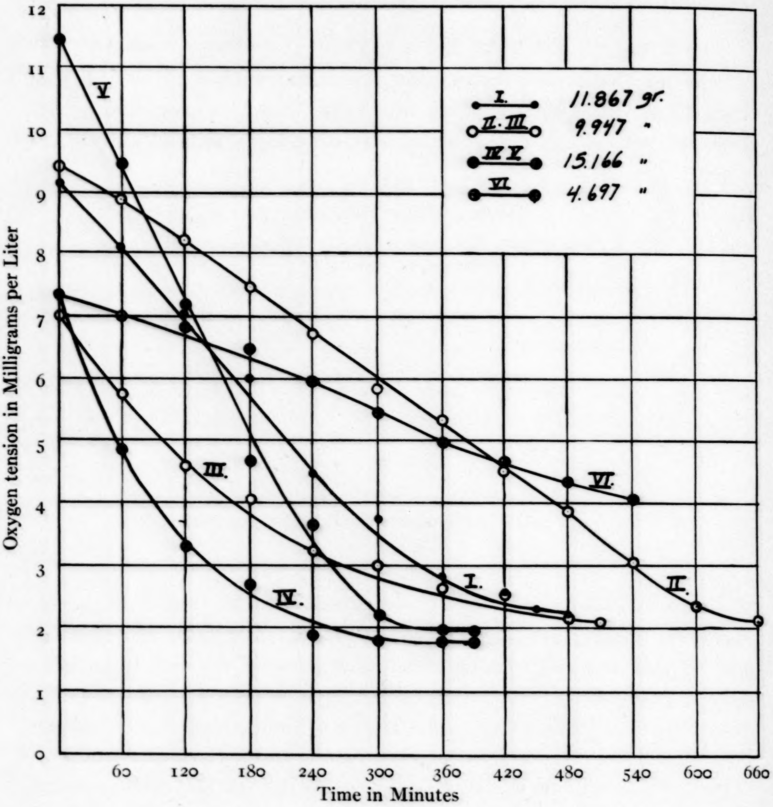

FIG. 4.—*Cambarus virilis:* Oxygen consumption in relation to oxygen tension. The same symbols are used for the same individuals.

Experiment II. Identical with the previous experiment except that two smaller crayfish were used and the experiment started at a higher tension of oxygen.—The total weight of these two animals was 9.947 gm. The oxygen content of the water was 9.40 mg. per liter at the start. The experiment was run for a period of 11 hours, at the end of which time the oxygen consumption had begun to diminish.

Graph II, Figure 4, indicated another straight-line relationship showing a practically complete independence of oxygen tensions over the entire range. In the following experiment the same individuals were again used, but this time they were started at a lower tension of oxygen.

TABLE XII

Cambarus virilis: EXPERIMENT II

2 different individuals. Total wt., 9.947 gr. pH, 7.8.
Temp., 21.5° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)
0	960	4.70	9.40
60	948	4.44	8.88
120	936	4.10	8.20
180	924	3.72	7.44
240	912	3.35	6.70
300	900	2.90	5.80
360	888	2.64	5.28
420	876	2.25	4.50
480	864	1.93	3.86
540	852	1.55	3.10
600	840	1.18	2.36
660	828	1.06	2.12

Experiment III. To determine the effect of diminishing oxygen tensions upon the rate of oxygen consumption of the same individuals used in the preceding experiment—but starting at a lower tension of oxygen.—A slight change is shown in Graph III, Figure 4. The two animals this time showed more or less dependence upon tensions; not to any great extent at first, but at tensions below 3.24 mg. of oxygen per liter. It occurred to the writer at this time that possibly the animals were not actually dependent upon the oxygen tension but were simply unable to keep up their normal rate of oxygen consumption because of too short a time allowed for adjustment. If this assumption were true, it should follow that larger individuals should be still more dependent if the same amount of water were used. Therefore, in the following experiment two larger crayfish were used in water from the same stock supply at approximately the same tension as in Experiment III.

TABLE XIII

Cambarus virilis: EXPERIMENT III

Same 2 individuals. Wt., 9.947 gr. pH, 7.8.
Temp., 21.5°–22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)
0	960	3.50	7.00
60	948	2.85	5.70
120	936	2.30	4.60
180	924	2.04	4.08
240	912	1.62	3.24
300	900	1.50	3.00
360	888	1.32	2.64
420	876	1.28	2.56
490	864	1.10	2.20
510	852	1.05	2.10

Experiment IV. To determine the effect of diminishing oxygen tensions upon the rate of oxygen consumption in larger crayfish, using the same volume of water as previously.—Two larger individuals, with a total weight of 15.166 gm., were used in water of nearly the same tension as in Experiment III. The results of Experiment IV are shown in Figure 4, Graph IV. A curve was obtained similar to No.

TABLE XIV

Cambarus virilis: EXPERIMENT IV

2 larger individuals. Wt., 15.166 gr. pH., 7.8.
Temp., 22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)
0	960	3.65	7.30
60	948	2.42	4.84
120	936	1.65	3.30
180	924	1.34	2.68
240	912	0.92	1.84
300	900	0.90	1.80
360	888	0.88	1.76
390	876	0.88	1.76

III, except that the rate of oxygen consumption was greater, as would be expected when using larger animals. In other words, a greater dependence existed when proportionately less oxygen was present. If this dependence were due to an insufficiency of water (and thus indirectly of oxygen), would it not be possible to overcome this dependence by using the same amount of water with a much higher oxygen content? With this question in mind, Experiment V was conducted.

Experiment V. To determine the effect of diminishing oxygen tensions upon the rate of oxygen consumption when high tensions of oxygen are present.—Oxygen from a tank was shaken in contact with the stock water until 11.40 mg. per liter were dissolved. The same two animals which showed a distinct dependence upon oxygen tensions of 7.30 mg. and less were placed in this supersaturated water. Here a decided change in relationship can be seen (Fig. 4, Graph V). Instead of the exponential type of curve obtained in Experiment IV, a straight line appears down to tensions of 2.24 mg. This indicates two things: first, that an independence exists above tensions of the preceding experiment, and, second, that when once started, an independence can exist to lower tensions than otherwise. Probably if the organism does not have enough available oxygen at the start it forms an oxygen debt which increases as the available oxygen grows less. If this statement be true, a small individual started at an average normal tension of oxygen should (if small enough

TABLE XV

Cambarus virilis: EXPERIMENT V

Same individuals as Experiment IV. Wt., 15.166 gr.

pH, 7.8. Temp., 22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)
0	960	5.70	11.40
60	948	4.74	9.48
120	936	3.58	7.16
180	924	2.32	4.64
240	912	1.82	3.64
300	900	1.12	2.24
360	888	0.98	1.96
390	876	0.95	1.90

and thereby having a lower respiratory rate) be independent over a considerable range of tensions. For the purpose of testing this hypothesis, the smaller of the two individuals used in Experiment II was selected, and Experiment VI started at a tension of 7.30 mg. of oxygen per liter.

Experiment VI. To determine if a small animal (whose needs for oxygen are not too great) can be independent of tensions upon which larger forms have been found to be apparently dependent.—The results of this test (Graph V, Fig. 4) bear out the aforementioned hypothesis and indicate that in the preceding tests, Experiments III and IV, a "dependence," as shown by the corresponding graphs, was due actually to an insufficiency of oxygen at the start of the experiments and was not a true dependence governed by the actual tension of oxygen. In other words, if more water had been used, or a higher tension of oxygen in the same amount of water, there would have been no need for an oxygen debt to exist.

TABLE XVI

Cambarus virilis: EXPERIMENT VI

1 individual. Wt., 4.697 gr. pH, 7.8. Temp., 22° C.

Minutes	Water Volume (Cc.)	Thiosulphate (Cc.)	Oxygen per Liter (Mg.)
0	960	3.65	7.30
60	948	3.50	7.00
120	936	3.40	6.80
180	924	3.22	6.44
240	912	2.95	5.90
300	900	2.72	5.44
360	888	2.46	4.92
420	876	2.34	4.68
480	864	2.18	4.36
540	852	2.05	4.10

DISCUSSION

With each type of animal used in the foregoing experiments respiration was found to be independent of oxygen tensions over a part of the range. The forms showing the least degree of independence were the crayfishes. In certain cases, curves were obtained showing that the oxygen intake was limited by the oxygen tension of the water, but this apparent dependence disappeared when water of a higher oxygen tension was employed, or when smaller crayfish were used in the same quantity of water. The explanation of this, no doubt, lies in the fact that an increase in the volume of water, while not actually increasing the oxygen tension, increases the amount of available oxygen so that, as the organism consumes oxygen, the tension of the gas diminishes more slowly, allowing the animal more time to regulate its respiration. Thus, considerable time must be allowed for this organism to adjust its respiration. Amberson *et al.* (1924) have shown that the respiration of the lobster, *Homarus*, and the sandworm, *Nereis*, is dependent upon the diminishing tensions when these tensions are lowered by the respiration of the animals themselves. It can be seen from their graphs of oxygen consumption that the oxygen tension was lowered to one-tenth that of the start in only 280 minutes in the case of the lobster and in 150 minutes in the case of the sandworms. It would be interesting to measure the respiration of these animals in a much larger volume of water to determine if this dependence still existed when a longer time was allowed. It seems that a very efficient regulatory mechanism would need to be present to enable a water-breathing form to adjust itself to tensions diminishing at such a rapid rate. These investigators considered this diminishing oxygen consumption as an adaptive regulation of metabolism to lowered oxygen tensions. I am prone to consider it a lack of metabolic regulation, for, if an animal is able to regulate its metabolism, we should expect to find it able to endure variations from normal without appreciable change in its own activity. It is not probable that an organism with a respiratory pigment should be unable to regulate its own respiration over any part of the range of oxygen tensions. Of course, a compensatory regulation of metabolism may require some time to be effective, as has been shown by the writer's experiments with caddis-fly larvae and crayfishes.

If the oxygen tension diminishes rapidly so that the oxygen present in the medium is not ample to supply the needs of the organism, an oxygen debt must be formed, which should increase in proportion to the rate at which the tension decreases, until a point is reached at which oxygen consumption ceases entirely. A truly dependent form would be an organism whose oxygen consumption remains proportional to the oxygen tension when the animal is kept for a long period of time in a medium of uniform tension. Such a dependence has been demonstrated by Hyman (1929) in the starfish, *Patiria*, and in the sea urchin, *Strongylocentrotus*. In all probability, with such organisms as these, no respiratory pigments exist, so that the oxygen consumption must depend upon the rate of diffusion of oxygen through the tissues. Thus a decrease in oxygen tension would cause a corresponding decrease in diffusion rate.

In all probability, a reserve supply of oxygen exists in the tissues of the animals used in the writer's experiments, since it has been shown that crayfish started at high oxygen tensions (Graph V, Fig. 4) could maintain a uniform rate of oxygen consumption until quite low tensions were reached, even when the tension diminished fairly rapidly.

CONCLUSIONS

1. Nymphs of the dragon fly, *Nasiaeschna penthacantha*, respire at a uniform rate in oxygen tensions from normal saturation of water to a certain level below which tension oxygen consumption grows less as the tension diminishes.

2. Free carbon dioxide in the water, as well as a lowered pH, or a combination of both these factors, stimulates the rate of oxygen consumption, at the same time enabling the nymphs to continue their consumption of oxygen to lower tensions than otherwise.

3. The larvae of the caddis fly, *Limnephilus rhombicus*, are normally independent of oxygen tensions above normal saturation of the water and below to a level of about 1.60 mg. of oxygen per liter.

4. If the oxygen tension diminishes too rapidly, the oxygen consumption of the caddis worms grows less in proportion to the diminishing tension.

5. Oxygen consumption of the crayfish, *Cambarus virilis*, is normally independent of high oxygen tensions and low tensions down to about one-fourth the normal equilibrium of air and water.

6. If the oxygen tension diminishes too rapidly, oxygen consumption of the crayfish also decreases proportionately.

7. Evidence indicates that a certain amount of oxygen is stored

by the tissues, and that an oxygen debt is formed if enough time is not allowed for the normal absorption process to take place.

8. In the three forms used, the presence of respiratory aids, such as pigments, has been indicated.

9. It is not probable that oxygen consumption in these forms behaves as a simple physical diffusion process.

10. All of the foregoing animals have the ability to regulate their respiratory metabolism over most of the range of oxygen tensions, if allowed sufficient time.

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THE INFLUENCE OF OXYGEN TENSION ON THE OXYGEN CONSUMPTION OF RANA PIPIENS LARVAE

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THE range of organisms studied as regards the relationship of oxygen tension to oxygen consumption has been considerable. The results obtained have in many cases been contradictory on closely allied species and have therefore given rise to conflicting conclusions and even generalizations for certain classes of organisms. An extended review of the literature is not appropriate to the present paper, and the reader is consequently referred to the review of Helff (1928) and especially to that of Hyman (1929) for a phylogenetic treatment of the work to date. Certain generalizations may be stated, however, which apply to the invertebrates and vertebrates as a whole. These generalizations may be stated as follows:

The invertebrates as a group exhibit a greater degree of diverse reactions as compared with the vertebrates. They can, however, be said to conform to one of three types of responses: Their oxygen consumption may be markedly dependent, or even proportional in some cases, to the oxygen tension. This condition has been shown to exist for *Anemonia*, *Sipunculus*, and *Actinia* (Henze, 1910), *Astacus* (Brunow, 1911), *Cassiopea* (McClendon, 1917), *Paramoecium* (Lund, 1921), *Homarus*, *Nereis*, *Limulus*, and *Calinectes* (Amberson *et al.*, 1924), *Anodonta* (Dakin and Catherine, 1925), *Caudina* (Nomura, 1926), and *Patiria* and *Strongylocentrotus* (Hyman, 1929). Conversely, certain invertebrates appear to be strikingly independent of the oxygen supply over considerable ranges of oxygen tension. As examples of this reaction, the work of Henze (1910) on *Eledone*, *Scyllarus*, *Carcinus*, and *Aplysia*, and of Moore *et al.* (1912), on *Asterias* and *Echinus*, may be cited. Finally, many invertebrates, although possessing good respiratory regulation over certain definite ranges of oxygen tension, appear to lose this independence above or below these limits. This is said to be true of *Limax*, *Tenebrio*, and *Lumbricus* (Thunberg, 1905), *Planaria* (Lund, 1921), *Palaemonetes* and *Loligo* (Amberson *et al.*, 1924), *Actacus* (Hée and Bonnet, 1925), and *Cambarus* (Helff, 1928).

The literature reveals a surprising conformity of results as regards the situation among the vertebrates. Here it would appear that, with few exceptions, oxygen consumption is apparently independent of the oxygen tension of the environment in both land and aquatic forms. As examples of this, the work of Winterstein (1908) on *Leuciscus*, Henze (1910) on *Coris* and *Sargus*, Moore *et al.* (1912) on *Blennius*, Amberson *et al.* (1924) on *Fundulus*, Schneider *et al.* (1924) on man, Dakin and Catherine (1925) on goldfish, and Burfield (1928) on plaice eggs may be cited.

A review of the literature as it applies to the vertebrates will reveal that the bulk of the work has dealt with the mammal or fish. Among those classes which have received lesser attention, the amphibians are included. Here what little work has been done has concerned itself with the respiration of the adult frog especially. The amphibian larvae have received practically no attention and, in fact, the writer is aware of but one piece of work (Dakin and Catherine, 1925) on the axolotl. Even here the conclusions have been based on experiments made on but two larvae. No data are given, but the results are indefinitely stated in a few sentences to the effect that the oxygen consumption of the axolotl is independent of the oxygen tension until the latter falls below a certain minimum (minimum point not stated), at which time the oxygen consumption is "profoundly affected."

The fact that only meager results on one urodele (a neotenous form) and no work on anuran larvae has been done would seem to justify a more thorough investigation on some larval amphibian. The larva of *Rana pipiens* has therefore been selected for the present work as being representative of native anura and likely to be productive of results typical for most, if not all, amphibian larvae.

The writer wishes to express his appreciation for the facilities tendered by the Iowa Lakeside Laboratory, Milford, Iowa, where the major portion of the experimental part of the present paper was carried out.

MATERIALS AND METHODS

The stock employed in all series tested was obtained from a small pond in the vicinity of Spirit Lake, Iowa, during the months of June, July, and August, 1929. This particular strain of *Rana pipiens* larvae was exceptionally suited for the work at hand in that the individuals were well developed and nourished when collected, and could be secured in large numbers for purposes of selection. Although this particular strain grew to the abnormally large size of 110–15 mm. before the onset of metamorphosis, only healthy specimens between 70 and 80 mm., total length, were selected for testing purposes. This obviated the criticism of subsequent results that changes in the metabolic rate due to the possible nearness of metamorphosis, as previously shown by Groebbels (1922), Abelin and Scheinfinkel (1923), and Helff (1926), might be operative.

All oxygen consumption tests were made by the Winkler method. The tests were made with lake water, the temperature throughout being maintained at 26 ± 1° C. Special care was taken in eliminating the usual factors, as far as possible, which influence respiration. In this regard, the larvae used were always starved for at least 48 hours preceding the initial oxygen consumption tests. The factor of muscular movement was largely eliminated by keeping the larvae in lake water at 26 ± 1° C. during the starvation period. When this treatment failed to remove excessive movement during the tests, the data of the latter were discarded. The weighing of the animals was also made following the completion of the last oxygen consumption test, in order not to excite the larvae, as might have been the case had they been weighed immediately preceding the tests.

The various oxygen tensions under which oxygen consumption was recorded were obtained by letting the larvae reduce the tension through their own respiration. Although this raises the objection that metabolic waste products, and especially CO_2 which tends to accumulate, might have influenced the results, subsequent data obtained when using large quantities of testing water gave evidence that the sum total of the accumulated waste products was never sufficient to effect the oxygen consumption of the larvae. Further details regarding the procedure for the various types of tests made will be given under the respective headings of the latter.

NORMAL VARIATION IN OXYGEN CONSUMPTION

Previous work had indicated that frog larvae exhibit considerable variation in oxygen consumption during successive periods of time. It was of prime importance, therefore, that preliminary tests be made to determine the magnitude of this variation characteristic of the particular strain to be utilized for the subsequent oxygen-tension work. The method of testing was briefly as follows: Five tests were made on each of 20 larvae for five consecutive 90-minute periods. The larvae were tested, individually, in order to determine possible differences between specimens as regards magnitude of variation. The tests were run in liter flasks with water having an oxygen content between 6.0 and 7.0 cc. per liter at the beginning of the testing periods. The variation in the oxygen tension of the water between any two of five consecutive periods during which an animal was tested did not exceed 4 per cent. In the majority of cases, however, this did not exceed 2 per cent. In either case, this variation was a negligible amount considering the wide range of oxygen tensions considered in relation to the main problem investigated. The actual testing procedure involved the emptying of the experimental flasks at the end of each 90-minute period and the filling with fresh water for the succeeding test. In this manner the oxygen tension and purity of the water was maintained quite uniform at the beginning of each testing period. The respiration of the larvae, of course, resulted in a drop in tension during the test, but this decline was subject to slight variation when comparing the results of the five consecutive tests, one with another, for any one larva.

The results of this series are represented in Table I. The data show that certain individuals exhibit considerable variation in oxygen consumption between successive tests, while the respiration of others is maintained quite uniform. There is, however, no evidence that prolonged testing results in a gradual decline in oxygen consumption, as has been observed by several workers on other organisms. This can be shown, statistically, by averaging the results of the 20 larvae tested for each of the five testing periods. This procedure is also productive, perhaps, of a fairer estimate of the average variation between any two of the five test periods. The greatest difference evidenced in this regard was between periods three and four, the latter being approximately 12 per cent higher than the former. It must be remembered, however, that this difference was generally exceeded when considering the results of any one individual.

OXYGEN CONSUMPTION AT VARIOUS OXYGEN TENSIONS

The degree of normal variation in oxygen consumption having been ascertained, it was next desired to determine the possible effect of various oxygen tensions on respiration. Tests on individual larvae were consequently made in the following manner: The larva to be tested was placed in a liter flask and the latter filled with water having a fairly high, but known, oxygen content. This was usually done early in the morning so that the entire testing of the individual could be obtained within one day. At the end of the first hour and a half period, a definite quantity of water was drawn from the flask and subsequently analyzed to determine the oxygen content existing at this time. The oxygen consumption of the larva for this period

TABLE I

NORMAL VARIATION IN OXYGEN CONSUMPTION OF TADPOLES

ANIMAL	CUBIC CENTIMETERS OF OXYGEN CONSUMED PER GRAM WEIGHT DURING CONSECUTIVE 90-MINUTE PERIODS				
	First	Second	Third	Fourth	Fifth
1	0.143	0.121	0.124	0.133	0.125
2	.113	.154	.083	.139	.110
3	.174	.217	.114	.149	.095
4	.104	.172	.094	.124	.104
5	.163	.180	.165	.188	.130
6	.159	.162	.126	.188	.164
7	.122	.112	.127	.106	.107
8	.105	.084	.093	.104	.093
9	.168	.134	.146	.145	.143
10	.120	.095	.069	.110	.080
11	.104	.131	.115	.147	.135
12	.118	.080	.121	.120	.138
13	.143	.127	.126	.146	.151
14	.125	.145	.122	.130	.127
15	.103	.114	.127	.144	.132
16	.154	.130	.143	.136	.135
17	.079	.102	.125	.112	.112
18	.090	.109	.123	.121	.132
19	.177	.164	.179	.153	.168
20	0.099	0.107	0.090	0.100	0.112
Average	0.128	0.132	0.121	0.135	0.125

could thus be determined. Immediately after removing the sample for analysis, a similar amount of water of approximately the same oxygen content was added to the experimental flask, and the latter stoppered. The added water had been previously prepared by allowing several larvae to slowly exhaust the oxygen content of a 4-liter bottle of water. This procedure was then repeated every hour and a half until the larva became asphyxiated or died. Each succeeding test was therefore made with water at an initially lower oxygen content resulting through the respiration of the animals themselves. In order to determine the exact oxygen tension at the beginning of each hour-and-a-half period, it was necessary, however, to calculate the sum total of the oxygen remaining in the flask after the sample had been drawn and the amount of oxygen contained in the water added to the flask. This of course was also necessary in order to determine accurately the oxygen consumption of the larva for the next hour and a half period.

The complete data for the 24 larvae, so tested, are represented in Tables II–VI, inclusive. A survey of the data presented there will show that in general the oxygen consumption of the larvae remains fairly constant over considerable ranges of oxygen tension. Variation, it is true, is quite apparent, although the majority of individuals show hourly differences in oxygen consumption typical of normal variation. The type of variation present is similar to that of normal variation, also, in that the increases and decreases between any two of the successive tests is quite unpredictable and follows no set rule. Within certain limits 15 individuals (Nos. 1, 2, 3, 4, 6, 7, 10, 11, 13, 14, 15, 16, 18, 19, and 20) showed a constant oxygen consumption within the normal range of variation. The respiration of eight others (Nos. 5, 9, 12, 17, 21, 22, 23, and 24), while in general showing a constant oxygen consumption, exhibited occasional decreases and increases which could not be accounted for by normal variation. One individual (No. 8) gave some evidence of a lowering in oxygen consumption, although it was not clearly correlated or proportional to the decreasing oxygen tensions.

The average oxygen content at the beginning of the tests of the 24 larvae tested, was 6.116 cc. per liter, while the average tension at which respiratory regulation ceased was approximately 1.287 cc. per

TABLE II

OXYGEN CONSUMPTION OF TADPOLES AT VARIOUS OXYGEN TENSIONS

HOURS TESTED	ANIMAL									
	1 (Wt., 2.9 Gm.)		2 (Wt., 4.8 Gm.)		3 (Wt., 3.5 Gm.)		4 (Wt., 3.5 Gm.)		5 (Wt., 5.7 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	5.959	......	5.959	......	5.959	....	7.784	......	7.784	......
1½	5.380	0.648	5.204	0.863	5.315	0.729	7.200	0.653	6.157	1.842
3	4.586	.724	4.454	0.702	4.721	.512	6.425	.823	5.822	0.388
4½	3.616	.933	3.396	1.062	3.956	.706	5.514	.932	4.307	1.656
6	2.822	.785	2.337	1.119	3.190	.591	5.104	.319	1.846	1.707
7½	2.029	.850	1.235	1.245	2.556	.726	4.329	.701	1.099	0.843
9	1.413	.666	1.014	0.269	2.041	.468	3.418	.887	1.670	0.617‡
10½	0.882	.597	0.838	0.224‡	1.446	.645	3.053	.325	......	......
12	0.706	0.231‡	......	......	0.978	.536	2.415	.651	......	......
13½	......	......	......	......	1.063	−0.060†‡	1.641	.832	......	......
15	......	......	......	......	......	....	0.957	.779	......	......
16½	......	......	......	......	......	....	0.638	0.418‡	......	......

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour-and-a-half periods tested represents the oxygen tension of the water at end of period.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of animal during the test period.

TABLE III

OXYGEN CONSUMPTION OF TADPOLES AT VARIOUS OXYGEN TENSIONS

HOURS TESTED	ANIMAL									
	6 (Wt., 5.2 Gm.)		7 (Wt., 5.2 Gm.)		8 (Wt., 4.9 Gm.)		9 (Wt., 5.6 Gm.)		10 (Wt., 3.7 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	8.766	......	8.766	......	8.766	......	5.529	......	5.529	......
1½	7.371	1.571	7.071	1.938	7.125	1.858	3.514	2.255	4.681	0.955
3	6.388	0.986	5.860	1.278	5.405	1.838	2.941	0.547	3.369	1.326
4½	5.160	1.241	4.204	1.777	4.177	1.298	1.528	1.506	2.450	0.938
6	4.362	0.759	2.739	1.583	3.194	1.022	1.261	0.307‡	1.662	0.848
7½	2.948	1.479	1.656	1.205	2.089	1.195	......	......	1.137	0.615‡
9	1.598	1.458	0.892	0.875‡	1.413	0.746	......	......	......	......
10½	0.983	0.703‡	......	......	1.106	0.368‡	......	......	......	......

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour-and-a-half periods tested represents the oxygen tension of the water at end of period.

‡ Indicates asphyxiation or death of animal during the test period.

TABLE IV

OXYGEN CONSUMPTION OF TADPOLES AT VARIOUS OXYGEN TENSIONS

Hours Tested	Animal									
	11 (Wt., 4.2 Gm.)		12 (Wt., 3.8 Gm.)		13 (Wt., 4.0 Gm.)		14 (Wt., 6.0 Gm.)		15 (Wt., 5.2 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	5.529	......	5.529	......	6.070	......	6.070	......	6.070	......
1½	4.356	1.341	4.200	1.504	4.844	1.371	4.681	1.564	4.762	1.495
3	2.813	1.628	3.238	0.962	3.694	1.204	3.281	1.506	3.220	1.689
4½	1.588	1.331	1.969	1.346	2.709	1.012	1.881	1.508	1.905	1.437
6	1.361	0.264	1.356	0.680	1.601	1.193	1.050	0.929	1.270	0.708
7½	1.044	0.373‡	0.700	0.756‡	0.944	0.741	0.875	0.230‡	1.088	0.229‡
9	......	......	......	......	0.780	0.220‡	......	......	......	......

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour-and-a-half periods tested represents the oxygen tension of the water at end of period.

‡ Indicates asphyxiation or death of animal during the test period.

TABLE V

OXYGEN CONSUMPTION OF TADPOLES AT VARIOUS OXYGEN TENSIONS

Hours Tested	Animal									
	16 (Wt., 5.0 Gm.)		17 (Wt., 5.5 Gm.)		18 (Wt., 5.0 Gm.)		19 (Wt., 4.5 Gm.)		20 (Wt., 5.0 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	6.070	......	4.047	......	4.047	......	4.047	......	4.047	....
1½	4.856	1.374	2.927	1.252	3.148	1.012	3.447	0.686	3.150	0.749
3	3.500	1.454	2.411	0.587	2.389	0.853	2.721	.813	2.144	.392
4½	2.144	1.475	1.550	.996	1.881	0.606	2.086	.743	1.356	.938
6	1.181	1.070	1.033	.666	1.050	1.008	1.497	.735	0.919	.593
7½	1.094	0.126‡	0.775	0.395‡	0.960	0.206	0.907	.757	1.006	−0.013†‡
9	......	......	......	......	0.735	0.348‡	0.726	0.295‡	......	....

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour-and-a-half periods tested represents the oxygen tension of the water at end of period.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of animal during the test period.

liter. This would indicate a range in respiratory regulation of at least from 108 to 23 per cent saturation, partial pressure, 26° C. Below the lower limit of respiratory regulation the oxygen consumption was found to drop markedly, and asphyxiation and death shortly occurred. Instances were recorded (see Nos. 3, 20, and 23) in which the asphyxiated or dead larva evidently gave up oxygen to the water instead of removing it from the latter.

TABLE VI

OXYGEN CONSUMPTION OF TADPOLES AT VARIOUS OXYGEN TENSIONS

HOURS TESTED	ANIMAL							
	21 (Wt., 3.7 Gm.)		22 (Wt., 3.8 Gm.)		23 (Wt., 5.0 Gm.)		24 (Wt., 4.3 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	6.113	....	6.113	....	6.113	....	6.113	....
1½	4.803	1.466	5.250	0.971	4.852	1.440	4.812	1.472
3	3.120	1.707	3.238	2.047	4.127	0.630	4.069	0.645
4½	2.367	0.713	2.756	0.426	2.177	2.070	1.925	1.270
6	1.887	.475	1.925	.856	1.224	1.038	1.444	0.507
7½	1.149	.785	1.269	.695	1.270	−0.061†‡	0.875	0.623‡
9	0.616	0.566‡	0.831	0.456‡	....	....	....	....

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour-and-a-half periods tested represents the oxygen tension of the water at end of period.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of animal during the test period.

OXYGEN CONSUMPTION IN LARGE AMOUNTS OF WATER OF LOW INITIAL OXYGEN TENSION

The results as indicated in Table II–VI, although giving evidence of respiratory regulation, were not as clear as might have been desired in determining the lower limit of regulation. This was due largely to the fact that the oxygen-tension drop was of such a magnitude during the 90-minute testing periods as to render the exact limiting tension only approximate. For this reason, and also since little data was evident regarding the oxygen consumption below this point, due to the onset of asphyxiation and death, further tests were devised in an effort to make these points more clear.

The resultant tests involved the same procedure as previously followed, with the exceptions that the larvae were tested hourly and in four times the usual amount of water (4-liter flasks being employed). The water utilized was first brought down to an oxygen content of 2.104–2.416 cc. per liter, by boiling, which represented a tension of between 37 and 43 per cent saturation, partial pressure, 26° C. The respiration of the larvae in four times the usual amount of water resulted in a much less gradual reduction in the total amount of oxygen present, and made possible the study of oxygen consumption at tensions more closely approximating one another than had previously been the case. The relatively lesser contamination of the water with metabolic waste products was also of help in determining the possible effect, if any, of this factor as applied to the results of the earlier tests. Finally, the oxygen consumption of larvae immediately preceding asphyxiation and death could be more easily studied.

Twenty larvae were individually tested in this manner, the complete data for which are represented in Tables VII–X, inclusive. The average tension marking the lower limit of respiratory regulation was 1.240 cc. O_2 per liter or about 22 per cent saturation, partial pressure, 26° C. This corresponded remarkably well with the 23 per cent limit ascertained in the preceding experiments, and tended to justify the conclusion reached as the result of those tests on this point.

The oxygen consumption above this lower limit, although showing a constancy within the normal range of variation for certain individuals, was in general more erratic for the majority of larvae tested than had been true of the earlier tests. A possible explanation for this may be found in the supposition that the larvae had not been fully adjusted to the initial low-oxygen tensions at which the tests were run, although all individuals tested were maintained in water for several hours having an oxygen content approximately the same as that used for the first hour test. A more plausible explanation may be suggested in that the respiratory regulation of larvae becomes less perfect at low tensions, and especially when nearing the lower limit of regulation.

The onset of asphyxiation, and death, was shown to be complete for all larvae within the next hour following the attainment of the

TABLE VII

OXYGEN CONSUMPTION OF TADPOLES IN 4 LITERS OF WATER AT LOW OXYGEN TENSIONS

HOURS TESTED	ANIMAL									
	1 (Wt., 4.3 Gm.)		2 (Wt., 3.6 Gm.)		3 (Wt., 3.3 Gm.)		4 (Wt., 3.4 Gm.)		5 (Wt., 2.8 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	2.104	....	2.104	......	2.174	......	2.104	......	2.111	......
1	1.930	0.681	1.932	0.678	2.047	0.491	2.014	0.353	1.885	0.935
2	1.583	1.361	1.808	.478	1.791	.971	1.891	.481	1.734	.695
3	1.430	0.585	1.603	.762	1.578	.803	1.644	.939	1.659	.348
4	1.082	1.343	1.480	.446	1.407	0.655	1.438	.780	1.470	.850
5	0.966	0.461	1.315	0.620‡	1.109	1.164‡	1.233	.793	1.395	.313
6	.812	.614	......	......	......	......	1.069	0.641‡	1.357	.202
7	0.889	−0.298†‡	......	......	......	......	......	......	1.282	0.281‡

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour periods tested represents the oxygen tension of the water at end of period.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of animal during the test period.

TABLE VIII

OXYGEN CONSUMPTION OF TADPOLES IN 4 LITERS OF WATER AT LOW OXYGEN TENSIONS

HOURS TESTED	ANIMAL									
	6 (Wt., 4.1 Gm.)		7 (Wt., 4.7 Gm.)		8 (Wt., 3.4 Gm.)		9 (Wt., 2.8 Gm.)		10 (Wt., 3.3 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	2.128	......	2.416	......	2.409	......	2.212	......	2.212	......
1	2.008	0.511	2.207	0.858	2.208	0.825	1.960	0.987	2.048	0.630
2	1.847	.717	1.956	1.069	1.967	1.034	1.847	.459	1.847	.785
3	1.767	.456	1.708	1.028	1.807	0.661	1.659	.743	1.767	.314
4	1.526	.930	1.333	1.486‡	1.646	.674	1.583	0.318‡	1.566	0.772‡
5	1.486	0.171‡	......	......	1.566	.325	......	......	......	......
6	......	......	......	......	1.405	0.578‡	......	......	......	......

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour periods tested represents the oxygen tension of the water at end of period.

‡ Indicates asphyxiation or death of animal during the test period.

TABLE IX

OXYGEN CONSUMPTION OF TADPOLES IN 4 LITERS OF WATER AT LOW OXYGEN TENSIONS

Hours Tested	Animal									
	11 (Wt., 4.0 Gm.)		12 (Wt., 2.5 Gm.)		13 (Wt., 3.2 Gm.)		14 (Wt., 3.2 Gm.)		15 (Wt., 3.4 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	2.212	......	2.212	......	2.332	......	2.369	....	2.154	......
1	1.999	0.839	1.967	0.962	2.207	0.652	2.128	1.105	1.981	0.898
2	1.708	1.161	1.847	.488	2.124	.501	2.048	0.492	1.817	1.156
3	1.458	0.999	1.686	0.638	1.999	.664	1.887	.807	1.666	0.595
4	1.291	0.688‡	1.325	1.436	1.791	.992	1.807	.493	1.333	1.340
5	......	......	1.285	0.171‡	1.666	.672	1.606	.967	1.166	0.777‡
6	......	......	......	......	1.499	.836	1.445	.813	......	......
7	......	......	......	......	1.458	.399	1.325	.712	......	......
8	......	......	......	......	1.250	.941	1.205	.599	......	......
9	......	......	......	......	1.083	.810	1.365	−0.473†‡	......	......
10	......	......	......	......	0.916	0.762‡	......	....	......	......

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour periods tested represents the oxygen tension of the water at end of period.

† Indicates the addition of oxygen to the water by the animal, instead of oxygen being removed.

‡ Indicates asphyxiation or death of animal during the test period.

TABLE X

OXYGEN CONSUMPTION OF TADPOLES IN 4 LITERS OF WATER AT LOW OXYGEN TENSIONS

Hours Tested	Animal									
	16 (Wt., 5.0 Gm.)		17 (Wt., 3.5 Gm.)		18 (Wt., 3.3 Gm.)		19 (Wt., 3.7 Gm.)		20 (Wt., 3.3 Gm.)	
	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed	Cc. O_2 per L.*	Cc. O_2 Consumed
0	2.127	......	2.203	......	2.203	......	2.203	......	2.203	......
1	1.992	0.630	1.896	1.026	2.097	0.777	1.975	0.998	1.972	0.916
2	1.817	.750	1.735	0.513	1.903	.756	1.716	.978	1.867	0.345
3	1.646	.656	1.370	.998	1.736	0.635	1.607	0.659	1.496	1.069
4	1.486	0.644	1.165	.780	1.397	1.001	1.251	1.071	1.398	0.495
5	1.245	1.037‡	1.013	0.501‡	1.256	0.536	1.098	0.876	1.207	.799
6	......	......	......	......	1.079	0.497‡	1.006	0.116‡	1.097	0.561‡

* The cubic centimeters of O_2 per liter for hour 0 represents the oxygen tension of the water in the experimental flasks at the beginning of the test. The cubic centimeters of O_2 per liter for all hour periods tested represents the oxygen tension of the water at end of period.

‡ Indicates asphyxiation or death of animal during the test period.

lower limit of respiratory regulation. Death for certain individuals was found to occur between the tensions of 18 and 21 per cent saturation, partial pressure, 26° C. For the majority of cases, however, it would seem that asphyxiation and death occurred practically as soon as the lower limit of respiratory regulation was reached or very shortly afterward. In other words, many individuals were found whose respiration was maintained practically constant, within the normal range of variation, until asphyxiation and death rapidly took place.

DISCUSSION

The results are indicative of a marked independence of oxygen consumption on oxygen tension between the range of 108 and 22 per cent saturation, partial pressure, 26° C. The tests as made in the present work were not designed primarily to ascertain the exact upper limit of regulation; and it is quite probable, therefore, that this point may lie considerably above 108 per cent saturation. In fact, the data of several individuals would indicate that the upper limit of respiratory regulation may be extended to at least 155 per cent saturation. As regards the lower limit of respiratory regulation, there can be little doubt but that an oxygen tension of 22 per cent saturation is the average for this particular strain. The fact that asphyxiation and death usually occurred at this tension, or slightly below, would indicate, furthermore, that respiratory regulation is quite adequate down to a point where the amount of oxygen is insufficient to sustain life. The results, therefore, are in agreement with those obtained by other workers on fish and vertebrates in general.

The possibility exists that the method of oxygen-tension reduction, through the respiration of the larvae tested, might tend to effect the oxygen consumption as waste products accumulated. Although CO_2 and other excretory products may have an effect on oxygen consumption, it is doubtful whether they ever attained a sufficient concentration to effect the respiration of the larvae. This conclusion is borne out by a comparison of the results of larvae tested in 4 liters of water with those obtained when only 1 liter was used. The concentration of waste products in the former case was logically but one-fourth that true for the latter method of testing, and yet the variation in oxygen consumption obtained was approximately the same or even somewhat more pronounced. Had the variation which occurred in the 1-liter tests been due in part to the accumulation of waste products, then the reduction of their concentration should have resulted in a lessening of this variation. That this was not the case, and also in view of the fact that the variation experienced was of the same magnitude as true for the normal variation tests where the water was changed each testing period, would indicate clearly, it seems, that waste products, as they occurred in the various tests, did not influence the latter's accuracy.

The normal variation in oxygen consumption prevalent in *Rana pipiens* larvae during successive hours is difficult of interpretation, although such rhythmic fluctuations are not unknown in other groups of organisms (a similar condition having been described, for example, by Helff [1928] in the crayfish). It is evident that even under constant oxygen tensions the oxygen intake is subject to considerable fluctuation, much more pronounced in some individuals than in others. Where oxygen consumption tests are made under changing oxygen tensions, the nature of the testing procedure and other factors may possibly be of serious consequence, as suggested recently by Hyman (1929). Normal fluctuation under constant oxygen tensions can only be explained, however, by a more thorough knowledge of the respiratory mechanism operative in the organism concerned. Whatever this mechanism may prove to be, it is evident that oxygen want and oxygen intake are not closely adjusted in *Rana pipiens* larvae. It would appear likely that a considerable depletion of the blood or intracellular oxygen reserve must take place before the respiratory mechanism functions to increase the oxygen intake. Whether this increased oxygen consumption is due to the functioning of the integument or gills is a question as yet unanswered. The fact that the larvae frequently liberated oxygen to the test water during asphyxiation and death would indicate that an oxygen reserve actually exists, being released in this case when the respiratory mechanisms fail to function properly under low oxygen tensions. The foregoing and other questions which have arisen during the course of the present work are to be more thoroughly investigated, and an attempt made to determine, particularly, the nature of the controlling respiratory mechanism present.

SUMMARY

The results of over 400 Winkler oxygen-consumption tests show that anuran larvae possess remarkable respiratory regulation throughout considerable ranges of oxygen tension. The following abbreviated results were obtained:

1. Twenty larvae were tested, individually, for five consecutive 90-minute periods. The average amount of variation in oxygen consumption was approximately 12 per cent between any two periods.
2. Twenty-four larvae were tested, individually, at 90-minute intervals under decreasing oxygen tensions produced by their own respiration. The larvae (average weight, 4.7 gm.) were found to possess respiratory regulation between approximately 108 and 23 per cent saturation, partial pressure, 26° C. Approximately the same variations in oxygen consumption were found to occur as previously determined under constant oxygen tensions.
3. To determine more accurately the lower limit of regulation, 20 larvae were tested, individually, as above, but at 1-hour intervals and in approximately four times the initial amount of water which had previously been reduced to about 40 per cent saturation. The lower limit of regulation was thus determined as approximately 22 per cent saturation.
4. Asphyxiation occurs very rapidly at or below the lower limit of regulation and is complete betweeen 21 and 18 per cent saturation.
5. The results are generally discussed and suggestions offered relative to the nature of the respiratory mechanism controlling oxygen consumption.

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THE NATURE OF THE PHOTOGENIC RESPONSE OF PHOTURIS PENNSYLVANICA

(Two figures)

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THE nature of the photogenic response is of considerable importance in regard to the problem of the mechanism of the cellular reactions involved in the response of a cell to an applied stimulus, for in this case two of the substances immediately involved in the production of light, namely, luciferin and luciferase, have been extracted and shown to produce light under proper conditions *in vitro*. Unfortunately, until recently, the analysis of this response has been impossible because of the instrumental difficulties involved in recording such a small rapidly changing light intensity. The purpose of the present investigation is to record and describe the response of the photogenic organ.

THE STRUCTURE OF THE PHOTOGENIC ORGAN[1]

The photogenic organs are located on the sternal plate of the sixth and seventh abdominal segments. In the male the organ extends over the entire sternal plate of both segments and is larger than that of the female, which is confined to a small area on each of the segments above. The organ lies directly beneath a transparent chitinous integument and is separated from it by a thin layer of cells, the hypodermis. Two layers of cells comprise the organ proper: the dorsal or urate layer which completely incloses the ventral photogenic layer except where it comes in contact with the hypodermis. Small branches of the tracheal system penetrate both the dorsal and photogenic layers.

The photogenic layer consists of three kinds of cells: the cells of the tracheal epithelium, the tracheal end-cells, and the densely granular photogenic cells. The cells of the tracheal epithelium are small and surround the tracheal tube. The tracheal end-cells are larger and are distributed over the dorsal and ventral surfaces of the photogenic layer. The photogenic cells form a layer one cell in thickness. They contain many granules of practically uniform size, usually aggregated in a dense zone about the nucleus with a less dense peripheral zone.

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[1] For literature citations on this subject to 1920, see Harvey (1920).

The existence of a nervous mechanism controlling the activity of the organ has been demonstrated experimentally by Lund (1911), and the presence of nerve fibers which supply the tracheal end-cells has been demonstrated histologically by Bongardt (1903) in *Lampyris splendidula*. There is no histological evidence, however, for the existence of nerve fibers passing to the photogenic cells.

METHODS

The record of the intensity of light produced during the response of the photogenic cells either to a maximum electrical stimulus of the nervous system or to a normal stimulus of central nervous origin is obtained as follows. The light emitted by the photogenic organ is received upon a caesium photo-electric cell; the output of this cell after one-stage amplification is measured by a Cambridge type string galvanometer whose deflections are recorded photographically. An analysis of the string deflection produced by a light intensity changing at a rate comparable to that of the photogenic cell serves to establish the linear nature of the relation between the intensity of light, incident upon the photo-electric cell, and the string deflection.[1]

The firefly is mounted upon platinum electrodes with the ventral surface about 5 mm. from the photo-electric cell. Both electrodes are inserted in the thorax; maximum stimuli from an induction coil are employed.

RESULTS

A group of typical records of the light intensity during the response, obtained by the foregoing method, are shown in Figure 1. The response of the photogenic cells of the male firefly (Fig. 1 *A*, *B*) is consistently of this shape and differs from that of the female firefly (Fig. 1 *C*) in that the latter in every case exhibits indications of the summation of two successive responses. The time between these responses and the magnitude of the second response is quite variable, and the resulting records therefore differ considerably. This difference between the male and female response may undoubtedly be attributed to differences in the distribution and innervation of the photogenic cells in the two sexes.

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[1] The apparatus used both for recording the light production and calibrating the string deflection was made available for this investigation through the kindness of Dr. Eric Jette and Dr. C. V. King, to whom the authors wish to express their gratitude. A description of the apparatus will be published by them elsewhere.

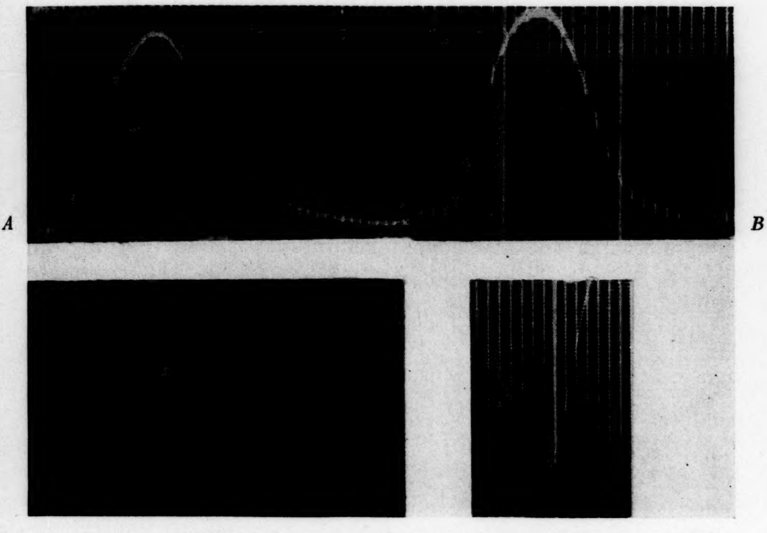

FIG. 1.—Photogenic response of *Photuris pennsylvanica*. *A*, male, 25° C.; *B*, male, 23° C.; *C*, female, 23° C.; *D*, initial portion of response of male at 23° C. See text. Records *B*, *C*, and *D* obtained under the same magnification. Time between ordinates, 0.0083 seconds.

The response of the male firefly shown in Figure 1 *A* is characterized by a rapid onset of light production, the light intensity subsequently increasing rapidly to a maximum in 0.063 seconds and then decreasing to zero in a total time of 0.153 seconds at 23° C. The shape of the initial portion of the response is illustrated clearly by Figure 1 *D*, which shows the first portion of a response equal in size to that of Figure 1 *A* but amplified by increasing the output of the photo-electric cell and the string sensitivity. From a point shortly after the maximum, the intensity diminishes as a logarithmic function of the time.

The magnitude of the response, as indicated by the light intensity produced at the maximum, varies widely with the number of stimuli and the frequency of stimulation; but the duration of the response remains constant in those cases where all of the cells in the photogenic organ are stimulated. The relation between stimulation rate and the magnitude of the response is shown in Figure 2, in which are plotted the data for a series of responses due to stimuli of central nervous origin and resulting from the application of a single electrical stimulus to an otherwise quiescent animal. This relation found for the rhythmic flashing of the organ fails to hold for the irregular flashing which occurs in an irritable preparation. In this case the response varies greatly in duration and magnitude. The examination of the active photogenic organ shows that the two types of responses have a different genesis, for, whereas in the former all of the cells are stimulated, in the latter only a few cells are involved, and these respond successively, giving the appearance of a light reaction creeping over the photogenic organ.

The constant duration of the response in the rhythmical flashing of the organ as opposed to the variable duration of the responses in the erratic flashes of an irritable preparation suggests that the character of the stimulus in each, although of central nervous origin, is entirely different. Since central nervous discharges in general consist of a series of impulses rather than one single impulse, it is probable that the response of a rhythmically flashing organ is caused by a volley of impulses from the central nervous system. Until such time as the nature of the stimulation is determined, it is not possible to state definitely whether the photogenic response recorded represents a summation of the responses of several stimuli or the response to a single stimulus.

DISCUSSION

It is impossible, on the basis of existing data, to present a critical analysis of the problem, namely, whether the photogenic response once started by the stimulation of the cell proceeds in the same way as the luciferin-luciferase reaction *in vitro* or whether this reaction is controlled to a great extent either by transformations in the cell associated with the responsive mechanism or by the diffusion of oxygen necessary for the reaction from the respiratory system. It may be pointed out, however, that the photogenic response as re-

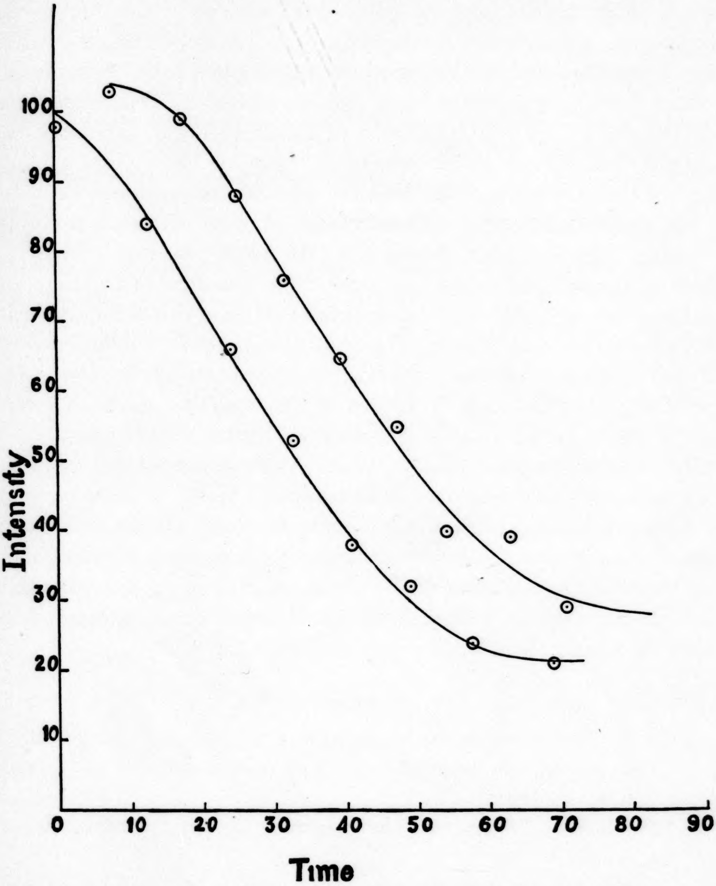

FIG. 2.—The magnitude of the response during a series of responses initiated by a single electrical stimulus. The time at which the first response occurs is plotted as zero, and subsequent responses are plotted at the time at which they occur after the preceding response. The data for the response of two different specimens are shown. Time scale: 10 units equals 0.833 seconds.

corded, although primarily dependent on a fundamental reaction of the luciferin-luciferase type, represents the summation of the different light intensities emitted by different regions of the photogenic organ, i.e., the greatest intensity in the vicinity of the tracheoles. Since this variation in the light intensity implies a difference in the velocity of the reaction in these regions, which can depend only on differences in the concentrations of either luciferin, luciferase, or oxygen, it is difficult to conceive of the response being a linear function of the fundamental reaction. In any case the regional variability in the light intensity emitted would interfere with such a simple relation. The fact that during the "die-away" portion of the response, the light intensity is a logarithmic function of the time, in conjunction with the fact that a similar relation exists for the luciferin-luciferase reaction *in vitro* (Amberson, 1922), might be construed to mean that the luciferin-luciferase reaction alone was determining the "die-away" portion of the response. Such an interpretation, to be justified, requires experimental corroboration.

At present the photogenic response, as recorded by this method, can be considered qualitatively as being the result of three factors: (1) the fundamental luciferin-luciferase reaction, (2) the successive stimulation of the cells in different regions of the organ and probably the repetitive stimulation of the whole organ, and (3) the variation in light intensity in different regions of the photogenic organ and possibly within the limits of a single cell.

SUMMARY

1. A method is described for recording a photogenic response.
2. The photogenic response of the male and female *Photuris pennsylvanica* is described.
3. The factors determining the genesis of the photogenic response are discussed.

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STUDIES ON THE BLOOD OF THE FETAL ALBINO RAT. II. THE HEMOGLOBIN CONTENT OF THE BLOOD AS RELATED TO THE DIFFERENTIAL ERYTHROCYTE COUNT

(Two Figures)

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PREVIOUS determinations of the hemoglobin content of the blood of the albino rat fetus were made by Nicholas (1928). He found a gradual increase during the latter part of the second third of the gestation period, and an extremely rapid rise in the early part of the third period of gestation. A slight fall in hemoglobin, which was followed by recovery, occurred shortly before birth. No erythrocyte counts were made. Recently Zeidberg (1929) has made a study of the hemoglobin content of the blood of the fetal rabbit from the twenty-second day of gestation until birth.

The albino rat was selected for the present study because of its short gestation period (21–22 days) and the consequent ease of obtaining a complete series of fetuses in sufficient numbers.

The mother was lightly anesthetized with ether, and a linea alba incision made in the abdomen. The fetuses were rapidly removed from the uterus as needed, with the membranes intact. Very little hemorrhage followed the separation of the placenta from the uterine wall. The amniotic sac was now opened, and the crown-rump length of the fetus obtained by means of a pair of fine calipers. The crown-rump length and the sex of each fetus was recorded. The jugular vein of the fetus was then opened, and blood samples for making the hemoglobin determination and the erythrocyte count were obtained. A smear was also made for staining, in order to obtain the differential count. One operator then prepared the hemoglobin sample by diluting it with 1 per cent HCl; while the other placed the second sample, diluted with Hayem's solution, in a mechanical agitator. A Bausch and Lomb hemoglobinometer No. 3600 was used in making the hemoglobin determinations.

Five fetuses from each litter were used, in most cases, and blood samples were then drawn from the jugular vein of the mother. Approximately 10 minutes elapsed from the time that the mother was placed under the influence of the ether until all samples had been diluted for hemoglobin determination or placed in the agitator. The mother remained alive throughout the procedure; and all fetuses were viable, as evidenced by spontaneous movement and attempts at respiration, even after the blood samples had been drawn.

TABLE I

SUMMARY OF AVERAGES FOR EACH LITTER SHOWING RELATIONSHIPS BETWEEN CROWN-RUMP LENGTH OF rat FETUSES, ERYTHROCYTES, AND HEMOGLOBIN CONTENT

Litter No.	No. of Fetuses	Crown-Rump Length (Millimeters)	Red Blood Corpuscles (per Cubic Millimeter)	Hemoglobin (Grams per 100 Cc.)	Percentage of Erythroblasts	Percentage of Normoblasts	Percentage of Basophilic Red Blood Corpuscles	Percentage of Eosinophilic Red Blood Corpuscles
8	5	15.4	694,000	5.52	9.96	3.92	13.26	72.66
7	5	18.4	1,118,000	6.60	7.48	3.38	10.42	78.72
1	5	18.8	1,362,000	5.56	7.60	3.06	12.08	77.26
9	5	22.6	1,716,000	8.76	2.04	1.60	6.28	90.08
5	5	23.4	1,788,000	9.20	1.80	1.40	5.80	91.00
6	5	28.4	1,870,000	9.98	0.80	1.00	6.28	91.92
17	10	29.1	1,929,000	9.46	0.40	0.47	10.84	88.29
12	5	29.4	2,095,000	10.08	0.58	0.48	10.62	88.32
14	5	29.8	2,176,000	9.62	0.12	0.40	4.92	94.82
16	5	33.4	2,078,000	9.54	0.10	0.18	5.42	94.30
2 13	10	33.6	2,168,000	9.55	0.11	0.30	6.06	93.03
10	5	34.0	2,065,000	8.90	0.26	0.92	10.74	88.08
3	5	34.2	2,380,000	8.14	0.04	0.22	13.14	86.60
15	5	34.4	2,206,000	9.68	0.00	0.20	4.86	94.94
18	5	35.6	2,316,000	9.66	0.00	0.12	4.84	95.04
4	5	43.0	2,594,000	10.78	0.00	0.04	2.52	97.44

The maternal erythrocyte count for the 17 litters averaged 7,245,000, and the hemoglobin content 10.63 gm. per 100 cc. of whole blood. No correlation could be established between these values and the age or number of the fetuses in utero.

Table I presents: (1) the number of fetuses used for the determinations and counts; (2) the average crown-rump length of the fetuses; (3) the average total erythrocyte count; (4) average hemoglobin content; (5) the average number of erythroblasts, normoblasts, and basophilic (polychromatophilic) and eosinophilic erythrocytes.

The stages of development have been expressed in crown-rump length only, since the fetal age cannot be accurately ascertained without determining the time of copulation. Even then, the error may be considerable, for the time elapsing between insemination and fertilization may vary considerably. Donaldson (1924) presents a table correlating the crown-rump length with the age of the fetus in days. Since, apparently, but a single litter was measured for each age represented, these figures were insufficient for our purposes. The determination of age by means of weighing the fetuses was impossible in this type of work, since too much time would be consumed in the process to insure the blood samples being in a fresh and normal condition when drawn.

Figure 1 shows graphically the total erythrocyte count and the hemoglobin content of the blood in fetuses of 15.4–43 mm. in length. The fetuses of 43 mm. length (litter No. 4) were exactly at term. Two young were born from this litter when the mother was immediately anesthetized, and samples drawn from the remaining young.

The hemoglobin content increases until a length of 18.4 mm. is reached, when it drops suddenly. The erythrocytes, however, continue to increase in number. A rapid recovery is seen in the hemoglobin until a length of 28.4 mm. is reached. Another slight and rapid fall follows with immediate recovery. This is succeeded by another slight drop. During this time the erythrocyte count steadily rises, being very rapid at the point at which the hemoglobin content falls.

At 33.6 mm. a marked fall in the hemoglobin occurs, while the erythrocyte count is seen to undergo fluctuation. After rapid recovery both erythrocytes and hemoglobin steadily increase until birth.

The fact that a fall in hemoglobin content was coincident with an increase in erythrocytes necessitated the making of differential erythrocyte counts of the blood of each fetus. One thousand cells were counted in each case, using smears prepared with Wright's stain. Ninety such counts were made. The cells were classified as erythroblasts, normoblasts, and basophilic and eosinophilic erythrocytes.

Figure 2 presents these counts graphically. The hemoglobin curve has been superimposed upon these graphs for purposes of reference.

Both erythroblasts and normoblasts decrease until they parallel each other. Very few are present after a length of 34.4 mm.

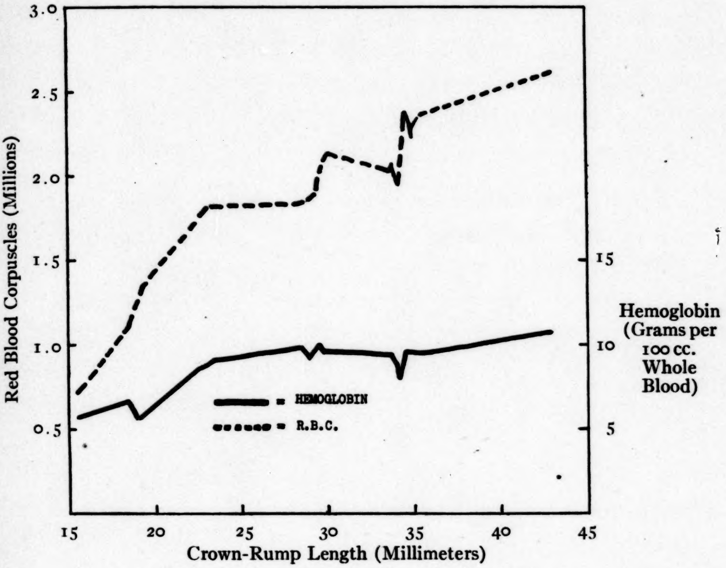

FIG. 1

In all cases the hemoglobin content rises and falls with the number of eosinophilic erythrocytes present. On the other hand, the basophilic erythrocytes rise and fall in inverse proportion to the eosinophilic cells.

Three rhythms are outstanding in all elements concerned: at approximately 18.8 mm., 29.4 mm., and 33.6 mm. It is suggested that these drops in the eosinophilic erythrocytes may be indicative of the duration of the life-span of these cells.

A comparison of our results with those obtained by Zeidberg presents numerous discrepancies, but an essential similarity is evident.

Zeidberg (1929) reports a steady rise in the hemoglobin of the fetal rabbit during the latter third of the gestation period. He observed none of the declines and subsequent rapid recoveries which our data exhibit. In the rat, the rise of hemoglobin during the last third of gestation is roughly 100 per cent. Zeidberg found a 50 per cent rise during a similar period in the rabbit.

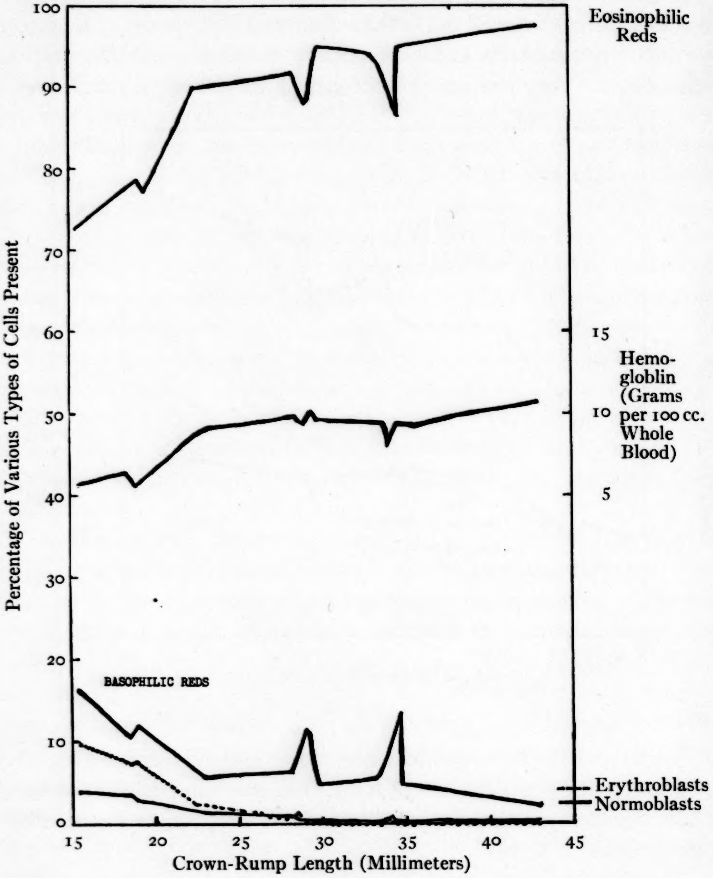

FIG. 2

TABLE II

Record of determinations made on each fetus

No.	Crown-Rump Length (Millimeters)	Sex	Red Blood Corpuscles (per Cubic Millimeter)	Hemoglobin (Grams per 100 Cc.)	Percentage of Erythroblasts	Percentage of Normoblasts	Percentage of Basophilic Red Blood Corpuscles	Percentage of Eosinophilic Red Blood Corpuscles
8-1	15	f	590,000	6.5	9.2	3.0	14.0	73.8
8-2	15	f	600,000	4.7	11.4	3.6	13.8	71.2
8-3	16	f	700,000	6.4	10.2	4.6	12.0	73.2
8-4	15	m	730,000	6.0	9.8	4.4	14.8	71.0
8-5	16	f	850,000	4.0	10.2	4.0	11.7	74.1
7-1	18	m	1,040,000	7.0	3.4	1.0	7.6	88.0
7-2	18	f	1,020,000	4.9	11.6	2.4	11.8	74.2
7-3	18	f	1,050,000	6.5	12.0	5.6	13.6	68.8
7-4	19	m	1,350,000	6.6	6.4	4.1	10.7	78.8
7-5	19	m	1,130,000	8.3	4.0	3.8	8.4	83.8
1-1	18	f	1,630,000	5.9	8.0	3.5	11.0	77.5
1-2	19	m	830,000	5.8	6.6	3.2	19.8	70.4
1-3	19	f	1,950,000	6.5	3.4	1.6	10.0	85.0
1-4	19	m	850,000	5.6	9.0	4.0	10.2	76.6
1-5	19	f	1,550,000	4.0	11.0	3.0	9.4	76.6
9-1	23	m	1,640,000	9.2	2.8	2.0	8.0	87.2
9-2	23	f	1,700,000	8.8	1.6	1.0	5.4	92.0
9-3	23	f	1,700,000	9.5	2.6	2.2	5.6	89.6
9-4	22	m	1,680,000	7.7	1.4	1.2	6.4	91.0
9-5	22	f	1,860,000	8.6	1.8	1.6	6.0	90.6
5-1	23	m	1,800,000	9.4	2.6	1.4	8.4	87.6
5-2	23	m	1,790,000	8.7	2.0	1.2	8.4	88.4
5-3	25	f	1,760,000	9.7	2.0	2.2	5.2	90.6
5-4	22	m	1,700,000	9.0	1.4	1.2	2.0	95.4
5-5	24	m	1,890,000	9.2	1.0	1.0	5.0	93.0
6-1	28	f	1,950,000	10.0	0.8	1.2	4.2	93.8
6-2	30	m	1,610,000	10.0	0.8	0.8	6.0	92.4
6-3	28	m	1,980,000	9.9	0.6	1.0	6.4	92.0
6-4	30	m	1,820,000	9.8	0.8	1.0	8.0	90.2
6-5	26	f	1,990,000	10.2	1.0	1.0	6.8	91.2
17-1	28	f	1,920,000	10.8	0.4	0.8	11.0	86.8
17-2	28	m	1,950,000	10.2	0.0	0.8	10.2	89.0
17-3	29	f	2,020,000	10.7	0.6	0.4	12.0	87.0
17-4	29	m	1,940,000	9.5	0.6	0.6	11.3	88.5
17-5	29	m	1,770,000	10.2	0.8	0.4	10.0	88.8
17-6	29	m	2,100,000	10.2	0.2	0.4	9.4	90.0
17-7	30	m	1,810,000	7.5	0.3	0.1	12.9	86.7
17-8	30	f	1,720,000	7.6	0.4	0.5	11.7	87.4
17-9	29	m	2,130,000	9.0	0.7	0.2	9.6	89.5
17-10	30	m	2,130,000	8.9	0.0	0.5	10.3	89.2
12-1	29	f	2,000,000	11.0	0.8	1.2	15.0	83.0
12-2	29	m	2,225,000	10.2	0.0	0.4	8.4	91.2
12-3	30	f	2,230,000	9.4	0.8	0.4	11.6	87.2
12-4	29	f	1,980,000	10.0	0.5	0.1	9.2	90.2
12-5	30	m	2,040,000	9.8	0.8	0.3	8.9	90.0

TABLE II—*Continued*

No.	Crown-Rump Length (Millimeters)	Sex	Red Blood Corpuscles (per Cubic Millimeter)	Hemoglobin (Grams per 100 Cc.)	Percentage of Erythroblasts	Percentage of Normoblasts	Percentage of Basophilic Red Blood Corpuscles	Percentage of Eosinophilic Red Blood Corpuscles
14-1	29	m	2,100,000	10.2	0.0	0.0	3.2	96.8
14-2	29	f	2,070,000	9.5	0.0	0.3	5.2	94.5
14-3	29	m	2,200,000	8.6	0.2	0.2	5.2	94.4
14-4	31	f	2,140,000	9.6	0.4	0.0	6.4	93.2
14-5	31	m	2,370,000	10.2	0.0	0.2	4.6	95.2
16-1	33	m	1,970,000	10.5	0.3	0.4	5.3	94.0
16-2	33	m	2,170,000	9.5	0.0	0.0	5.2	94.8
16-3	33	f	2,180,000	8.8	0.0	0.2	6.4	93.4
16-4	33	f	1,970,000	10.2	0.2	0.3	5.7	93.8
16-5	35	m	2,100,000	8.7	0.0	0.0	4.5	95.5
2-1	33	f	1,750,000	9.9	0.0	0.4	8.6	91.0
2-2	34	m	2,460,000	11.0	0.2	0.3	7.2	92.3
2-3	33	f	1,740,000	10.2	0.3	0.1	4.8	94.8
2-4	34	m	2,400,000	8.7	0.0	0.0	8.0	92.0
2-5	34	f	1,900,000	7.9	0.2	0.5	5.6	93.7
13-1	32	f	2,410,000	12.3	0.1	0.4	4.2	95.3
13-2	33	f	2,350,000	9.2	0.0	0.2	5.0	94.8
13-3	34	m	1,980,000	7.5	0.2	0.5	6.1	93.2
13-4	35	m	2,350,000	9.6	0.0	0.3	5.7	94.0
13-5	34	m	2,340,000	9.2	0.1	0.3	5.2	94.3
10-1	35	m	1,270,000	6.5	0.0	1.3	15.2	83.5
10-2	35	f	2,180,000	8.2	0.3	1.0	12.5	86.2
10-3	34	f	2,130,000	10.1	0.0	0.5	7.8	91.7
10-4	34	f	1,990,000	9.1	0.1	0.8	9.2	89.9
10-5	32	f	1,960,000	8.1	0.9	1.0	9.0	89.1
3-1	33	f	2,560,000	8.4	0.0	0.2	18.3	81.5
3-2	35	f	2,290,000	7.0	0.0	0.0	9.2	90.8
3-3	33	f	2,120,000	7.5	0.0	0.0	12.1	87.9
3-4	36	m	2,350,000	8.9	0.2	0.8	11.2	87.8
3-5	34	f	2,580,000	8.9	0.0	0.1	14.9	85.0
15-1	34	f	2,350,000	10.4	0.0	0.2	4.0	95.8
15-2	35	m	2,220,000	10.3	0.0	0.0	4.5	95.5
15-3	32	f	1,990,000	8.5	0.0	0.5	5.6	93.9
15-4	36	m	2,350,000	11.0	0.0	0.0	5.8	94.2
15-5	35	f	2,120,000	8.2	0.0	0.3	4.4	95.3
18-1	36	m	2,360,000	10.3	0.0	0.0	4.6	95.4
18-2	35	m	2,300,000	8.8	0.0	0.1	5.2	94.7
18-3	35	f	2,340,000	8.5	0.0	0.2	5.4	94.4
18-4	37	f	2,290,000	11.1	0.0	0.1	4.2	95.7
18-5	35	f	2,290,000	9.6	0.0	0.2	4.8	95.0
4-1	44	f	2,410,000	9.7	0.0	0.2	2.0	97.8
4-2	42	m	2,560,000	9.8	0.0	0.0	3.6	96.4
4-3	43	m	3,010,000	10.7	0.0	0.0	2.2	97.8
4-4	43	f	2,640,000	11.5	0.0	0.0	2.7	97.3
4-5	43	m	2,350,000	12.2	0.0	0.0	2.1	97.9

A closer comparison is seen in the case of the basophilic red cells. In the rabbit these decrease from 28 to 4 per cent. Our counts show a comparable reduction from 10 to 2 per cent.

Table II contains all of the data on each fetus studied. The reader is referred to this table for data not summarized above.

CONCLUSIONS

1. The concentration of the hemoglobin in the blood of the fetal albino rat was determined from 15.4 mm. in crown-rump length until birth (43 mm.).

2. Decreases in the hemoglobin content followed by rapid recoveries were observed at the end of the second third of gestation, at the beginning of the third period of gestation, and at the middle of the latter period.

3. The fluctuations in the hemoglobin content of the blood occur coincidently with similar changes in the number of eosinophilic erythrocytes present.

4. Erythroblasts and normoblasts steadily decrease in the blood of the fetal albino rat until they become numerically insignificant at the beginning of the third period of gestation.

5. There is no correlation between the total erythrocyte count or the hemoglobin content of the blood of the pregnant albino rat and the number or size of the fetuses in utero.

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AN EXPERIMENTAL STUDY OF THE OPSONINS OF THE BLOOD. I. EFFECT OF BILATERAL ADRENALECTOMY[1]

(One figure)

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SINCE the time of Metchnikoff it has been known that animal cells, prinçipally the polymorphonuclear leucocytes, are able to engulf and destroy invading organisms. This phagocytic action has been recognized as one of the important defenses against infection. Metchnikoff, as research progressed, was forced to modify his original theory of phagocytic action; and he showed that there was some substance in the blood which served to facilitate the phagocytic action of the leucocytes. Wright and Douglas (1904) showed more clearly the rôle of the serum in promoting phagocytosis. This substance in the serum, which facilitates the phagocytic action of the cells, was termed, by them, "opsonin"; and they devised a method for measuring the opsonic activity of blood serum. The exact nature of this opsonin is not known, and knowledge as to what tissue, or tissues, elaborate this substance is completely lacking.

The means by which the opsonins bring about phagocytosis is not, as yet, completely understood. The work of Fenn (1921*a*, *b*) tends to indicate that opsonins effect a change in the surface relationships between the leucocyte and the particle to be engulfed. Unless the particles can come in contact with the leucocyte and can "stick to" the cell, no engulfment can take place; and he has shown that the degree of phagocytosis, other conditions remaining constant, is proportional to the number of collisions between particles and cells. Mudd, Lucke, McCutcheon, and Strumia (1929) found

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[1] I wish to acknowledge my indebtedness to Professor W. W. Swingle for suggesting the problem and for his interest during the progress of the work. I wish, also, to express my appreciation to Dr. B. Lucke, of the University of Pennsylvania, for certain suggestions as to experimental methods.

that when bacteria were sensitized with serum several bacteria-leucocyte relationships were altered. There was an increase in cohesiveness, a decrease in surface electrical potential difference, and an increased phagocytosis. This third phenomenon may well be a result, in part, of changes in the first two factors.

The foregoing paragraphs will serve to outline briefly the known facts and the present theories in regard to the rôle of the opsonins in facilitating phagocytosis.

Many investigators, in endeavoring to discover the functional significance of the suprarenal cortex, have tended to show that there is some connection between the functional efficiency of the suprarenal cortex and the efficiency of an animal's resistance to infection. The results of such experiments have been far from conclusive. Investigators have disagreed as to results, and the conclusions drawn from the experimental data are open to criticism because most of the experimental animals used by these investigators were rats and rabbits. It is extremely difficult, almost impossible, to remove the whole of the adrenal cortical tissue from these animals. Accessory nodules of cortex are often present, and this amount is sufficient to maintain the animals in normal health. Some investigators have used rats two and three months after the supposed removal of the entire adrenal system. Such animals were entirely normal, as far as could be observed; and this would indicate that there was present, in the animal, a sufficient amount of functional cortical tissue to maintain a normal hormonic balance in the body. It seems difficult to draw conclusions as to the function of the suprarenal capsules, the cortical part in particular, from experiments in which an unknown, but considerable amount of accessory cortical tissue is undoubtedly present in the body of the experimental animal at the time of the experiment.

Various factors of the defense mechanism have been studied, and a few of these experiments will be briefly mentioned. As early as 1903 Oppenheim and Loeper stated that rats, ordinarily immune to diphtheria toxin, were susceptible after having been adrenalectomized. Later, in 1914, Marie and Morax claimed that suprarenalectomized guinea pigs showed a decreased resistance to tetanus toxin. More recently Scott (1924, 1927) has investigated the effect of adrenalectomy upon the resistance of rats to various toxins. In his earlier paper he states that the resistance of rats to bacterial intoxications is greatly decreased following double adrenalectomy. He believes that this decreased resistance is due to an insufficiency of the adrenal cortex. In his later paper he shows that adrenalectomized rats have an increased susceptibility to histamine, and he proposes that this susceptibility be used as a biological test for deficient cortical function. He used animals relatively soon after the removal of the adrenal glands and before a marked hypertrophy of the accessory cortical tissue could have taken place. The experimental animals, nevertheless, appeared perfectly normal and showed no evidences of deranged metabolic or hormonic balance.

Jaffe and Marine (1923) stated that adrenalectomized rats, taken before there was opportunity for appreciable amount of cortical hypertrophy, would succumb to one-twentieth of the dose of standard typhoid vaccine which normal rats would withstand. They, in agreement with most investigators, believe that the cortical portion of the adrenal gland is the important physiological factor in these experiments.

Other investigators have reported similar results; but in some of these experiments the animals have survived double adrenalectomy several weeks, sometimes months, and would have compensated for the original cortical insufficiency by a functional hypertrophy of the accessory tissue.

All investigators do not agree that a decrease in resistance follows the removal of the suprarenal capsules. Rogoff and Ecker (1926) criticized the work of Marie and Morax (1914), Jaffe and Marine (1923), and others, and stated that from their experiments they conclude that there is no foundation for statements that partial or complete adrenalectomy results in increased susceptibility. They used rats as the experimental animals and tetanus toxin as the substance injected.

It would seem, therefore, that the relation of the adrenal cortex to the defense mechanism should be studied in some animals which have no accessory cortical tissue. Under such conditions the results would not be influenced by the presence, in the experimental animal, of varying amounts of functional cortical tissue.

The animal best suited to such experiments would seem to be the cat or the dog. As cats could be kept in the laboratory with greater ease than could dogs, the former animal was used in this study. The cat has been used by many workers in studying the functional significance of the suprarenal cortex, and these experiments have shown that a very low percentage of these animals have any accessory cortical tissue. As the life-span of adrenalectomized cats is from 6 to 8 days, it would be impossible, because of the lack of time, to carry out immunity experiments following the removal of the glands. Studies of the susceptibility to specific toxins are rather difficult to control accurately, and comparable results are sometimes impossible to obtain in a series of experiments extending over any considerable time. It seemed best to study the opsonic activity of the blood of completely adrenalectomized cats. This factor of the defense mechanism can be accurately determined; and the method of determination, as outlined elsewhere in this paper, makes it possible to obtain comparable results in a long series of experiments.

Experiments were therefore carried out to determine if the removal of the adrenal glands resulted in any change in the opsonic activity of the blood serum of such operated animals.

MATERIALS AND METHODS

In carrying out an experiment to determine the opsonic activity, three substances are necessary; blood serum, leucocytes, and an antibody, i.e., a substance to be ingested by the cells. These three substances were obtained as follows:

The serum was obtained by cardiac puncture, and about 5 cc. of blood was withdrawn and transferred to a 15-cc. centrifuge tube. After the blood had clotted, the serum was separated from the cells by centrifuging. The serum was removed with a pipette and used in the experiments.

The leucocytes are obtained, free from all serum and red cells, by the method developed by Hamberger and used and reported by Mudd, Luke, McCutcheon, and Strumia (1929). The cat, from which the cells are to be obtained, is injected, intraperitoneally, with 200–300 cc. of 0.9 per cent NaCl. About 3 hours later a considerable quantity of the fluid can be recovered by puncturing the peritoneal cavity with a large-bore needle. The fluid flows spontaneously and is received in a flask containing a 1 per cent solution of sodium citrate in 0.9 per cent NaCl. The citrate prevents clotting and clumping of the cells. This peritoneal exudate is centrifuged at a slow rate of speed, and the supernatant liquid removed. The cells are washed with 0.9 per cent NaCl, recentrifuged, the fluid removed, and the cells suspended in a small amount of 0.9 per cent NaCl. This is the suspension of leucocytes used in the experiments. Two hundred cubic centimeters of peritoneal exudate will yield about 2 cc. of cells, 95 per cent of which are polymorphonuclear leucocytes.

Collodion particles were used as the substance to be injected by the cells. This substance is ideally adapted for use in opsonic activity experiments, as known dilutions of the antibody can be obtained at any time and the particles, being acid fast in staining reaction, can be easily identified when within the leucocyte. The suspension of collodion particles was prepared by the method of Loeb (1922). The suspension thus obtained was used as a stock solution. It keeps indefinitely, and known dilutions can be prepared at any time for use in an experiment.

The opsonic activity of the samples of serum was determined by the method perfected by Robertson, Woo, and Cheer (1924). This method gives results which are more accurate than can be obtained by the modifications of the older Wright method of opsonic-index determination. Fenn (1921*b*) has shown that the activity of any phagocytic system is proportional to the number of contacts made between particles and leucocytes. The method of determining the opsonic activity as used by Robertson and his co-workers (1924) gives every opportunity for contact between antibody and cells.

It has been shown by Meakins (1909) that accurate results can be obtained in opsonic activity determinations only when serial dilutions of the serum are made. This fact has been well established but often disregarded. In this study the serum was diluted with 0.9 per cent NaCl in the following dilutions: 1:4, 1:16, 1:64, 1:256, 1:1024 (approximately 1:1,000). These five dilutions, together with the undiluted serum, were used in setting up an experiment.

Small, hard-glass test tubes (12×35 mm.) are used; and into each tube is placed $\frac{1}{10}$ cc. of serum or its dilution, $\frac{1}{10}$ cc. of collodion suspension, and $\frac{1}{10}$ cc. of leucocyte suspension. The tubes are then stoppered with sterile corks and agitated for 15 minutes.

The mixer, or agitator, is a modification of the instrument described by Robertson, Woo, and Cheer (1924).

At the end of 15 minutes of mixing, the tubes are removed from the mixer and spreads of the contents of each tube made upon clean dry slides. The smears are made in the usual manner; a drop of the leucocyte-collodion-serum mixture is placed upon the slide, and the spread is made by drawing a second slide, the corners of which have been filed off, along the first slide. This results in an even film of material upon the surface of the first slide. If shrinkage and deformation of the leucocytes is to be prevented, the smears must be rapidly dried. This is done by placing the slides, face up, upon a warm electric hot-plate over which a current of air is passing. The dried slides are placed, face down, upon racks and exposed to the fumes of 40 per cent formalin for 5 minutes. This serves to fix the smear, and the formaldehyde vapors are removed from the slide by passing the flame of a Bunsen burner over the smears. The slides are then ready to be stained.

The usual acid-fast staining technique is used. The slides are placed in staining racks and stained in hot Ziehl-Neelsen carbolfuchsin for 10 minutes. The stain was kept at a temperature of 65°–70° C. during the staining process. The slides are washed in running water and then decolorized in 10 per cent sulphuric acid for about 10 seconds. This completely decolorizes the cells but leaves the collodion particles stained a brilliant red. The slides are again washed and counterstained with methylene blue for 10 seconds. After being washed and dried, the slides are examined under the highest power of the microscope.

Between 100 and 200 cells are counted on each slide, and the number of cells which have engulfed collodion particles determined. The results are expressed in terms of percentage of phagocytic cells. Due to the color contrast between the collodion particles and the leucocytes, there is never any doubt as to whether or not the cells have ingested the antibody. Only isolated cells are counted; clumps of cells are completely ignored.

As stated above, cats were used as the experimental animals. Unilaterally adrenalectomized cats were allowed to completely recover from the first operation before the second gland was extirpated. At least 7 days was allowed for this recovery to take place. The operated animals were kept in heated rooms the temperature of which was thermostatically controlled.

Following the second operation the animals were bled at frequent intervals, and the opsonic activity of the serum was determined by the method outlined above. At the same time, blood was drawn from a normal cat to serve as the control for the experiment.

RESULTS AND DISCUSSION

The results of these experiments can best be shown in table form.

The complete protocols of three typical experiments are given in Table I. In this table, and in those to follow, the results are expressed in terms of percentage of phagocytic cells. Each dilution of experimental serum is controlled by a like dilution of normal serum and the amount of spontaneous phagocytosis taking place in the phagocytic system is measured by setting up one or more test tubes containing $\frac{1}{10}$ cc. of 0.9 per cent NaCl in place of the serum.

The change in the opsonic activity of the blood following bilateral adrenalectomy can be shown graphically. In the curve shown in Figure 1 the opsonic activity of the serum has been plotted against time following the removal of the second adrenal gland. The experimental data obtained from 13 animals has been grouped together in plotting this curve; and the normal opsonic activity was arbitrarily taken as 100 per cent, and all the experimental figures were corrected to agree with this standard. As this study was not intended to be statistical, no attempt was made to obtain sufficient mathematical data to enable a smooth curve to be drawn. This curve, however, does show the trend of the change in opsonic activity of the blood following the complete removal of the suprarenal glands.

It is evident from the results shown above that the complete removal of the adrenal glands results in a decrease in the opsonin content of the blood. This decrease becomes more marked following the onset of the symptoms of adrenal insufficiency.

Experiments were next performed to check the possibility that the drop in opsonic activity might be due to metabolic disturbances incidental to the adrenal insufficiency rather than to the specific lack of the adrenal glands.

The symptoms which are characteristic of the conditions arising from the removal of the kidneys are very similar to those symptoms observed in the terminal stages of adrenal insufficiency (Swingle, 1928). The animal becomes weak, staggers when endeavoring to walk, and sways when standing still. This weakness gradually increases, and the animal sinks into a semicomatose condition and dies without regaining consciousness. The changes which occur in several of the blood constituents following adrenalectomy and

TABLE I

EFFECT OF BILATERAL ADRENALECTOMY UPON THE OPSONIC ACTIVITY OF THE BLOOD SERUM

Cat No. 4. Maltese, female. Right gland removed 1/27/30. Weight, 1,960 gm. Left gland removed 2/4/30. Weight, 1,950 gm. The cat was bled at the times given below, and the opsonic activity of the blood serum determined. Results are expressed in percentage of phagocytic cells.

	SERUM DILUTIONS (RATIO)					
	Undiluted Serum	1:4	1:16	1:64	1:256	1:1,000
Determination 1						
Cat bled 2/6/30, 2:15 P.M.						
Opsonic activity	60	51	39	25	21	15
Control serum	78	61	50	37	23	17
Spontaneous phagocytosis	11	11	11	11	11	11
Determination 2						
Cat bled 2/9/30, 2:30 P.M.						
Opsonic activity	36	25	17	13	15	12
Control serum	80	66	52	40	27	19
Spontaneous phagocytosis	14	14	14	14	14	14
Determination 3						
Cat bled 2/11/30, 9:50 A.M.						
Opsonic activity	22	15	12	15	11	14
Control serum	76	62	49	33	25	16
Spontaneous phagocytosis	13	13	13	13	13	13

Cat died 2/11/30, 4:30 P.M. Life-span, 7 days.

TABLE I—*Continued*

Cat No. 6 Black and white, male. Right gland removed 2/4/30. Weight, 2,100 gm. Left gland removed 2/13/30. Weight, 2,200 gm. Bled as shown below.

	SERUM DILUTIONS (RATIO)					
	Undiluted Serum	1:4	1:16	1:64	1:256	1:1,000
Determination 1						
Cat bled 2/14/30, 2:30 P.M. Animal was normal						
Opsonic activity	76	65	51	39	27	18
Control serum	81	68	53	39	29	18
Spontaneous phagocytosis	16	16	16	16	16	16
Determination 2						
Cat bled 2/16/30, 2:00 P.M. No symptoms						
Opsonic activity	45	33	20	16	13	11
Control serum	77	63	49	37	24	16
Spontaneous phagocytosis	14	14	14	14	14	14
Determination 3						
Cat bled 2/19/30, 1:30 P.M. Marked symptoms						
Opsonic activity	20	16	12	9	12	13
Control serum	70	58	40	29	20	11
Spontaneous phagocytosis	10	10	10	10	10	10

Cat died 2/19/30, 6:00 P.M. Life-span, 6 days.

TABLE I—*Continued*

Cat No. 7. Yellow tiger, male. Right gland removed 3/3/30. Weight, 1,620 gm. Left gland removed 3/14/30. Weight, 1,600 gm. Normal recovery. Bled at time shown below.

	Serum Dilutions (Ratio)					
	Undiluted Serum	1:4	1:16	1:64	1:256	1:1,000
Determination 1						
Cat bled 3/16/30, 2:00 P.M. Cat showed no symptoms						
Opsonic activity	62	50	39	28	20	16
Control serum	78	63	51	37	25	17
Spontaneous phagocytosis	15	15	15	15	15	15
Determination 2						
Cat bled 3/18/30, 2:15 P.M. No symptoms						
Opsonic activity	39	25	18	15	14	16
Control serum	80	67	53	40	28	17
Spontaneous phagocytosis	16	16	16	16	16	16
Determination 3						
Cat bled 3/22/30, 1:30 P.M. Showed marked symptoms of adrenal deficiency						
Opsonic activity	17	13	10	12	13	11
Control serum	72	59	43	30	21	12
Spontaneous phagocytosis	12	12	12	12	12	12

Cat died 3/22/30 about 8:00 P.M. Life-span, a little over 8 days.

nephrectomy are also similar. Marked symptoms following adrenalectomy or nephrectomy do not become evident until the last 24 or 36 hours of the life of the animal. During the latter stages of both conditions the animal refuses food. Because of this similarity between the metabolic changes and the symptoms arising from complete nephrectomy and those following complete adrenalectomy, the opsonic activity of the blood of completely nephrectomized cats was studied.

Nephrectomized animals have been used before as controls for experiments concerned with adrenal function. Gottsman and Gottsman (1926), in relation to resistance studies, reported that the life of nephrectomized rats was not affected by the same dose of typhoid vaccine which regularly shortened the life of adrenalectomized rats.

The technique of operation in the removal of the kidneys is essentially the same as that in removing the adrenals. As is the case in adrenalectomy, the kidneys are removed separately and at an interval of at least 7 days. The survival period of completely nephrectomized cats is from 4 to 6 days.

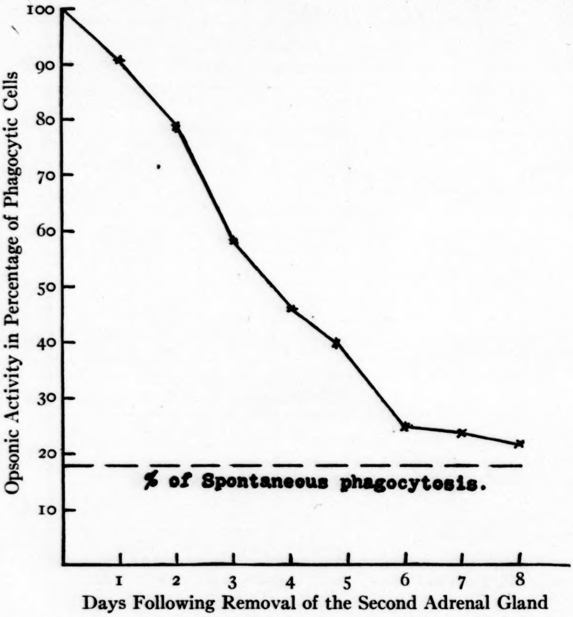

FIG. 1.—Change in opsonic activity following the removal of the adrenal glands

Table II shows a typical protocol of such a nephrectomized animal. The results are expressed in the same manner as in Table I.

From experiments performed with cats suffering from complete nephrectomy, it is evident that no drop in opsonic activity follows the removal of the kidneys in spite of the fact that the animals, during the last 36 hours of their life, show marked symptoms of a greatly deranged metabolism.

The next question is: Is the change in opsonic activity due to lack of the cortical portion or of the medullary portion of the adrenal gland?

Previous workers—Scott (1924, 1927), Jaffe and Marine (1923), Gottsman and Gottsman (1926), and others—believe that it is the cortical portion of the adrenal gland which is concerned in the maintenance of the normal resistance of an animal. No definite proof of this relationship has, however, been offered. It has been impossible to remove all of the medullary tissue and leave the cortical tissue, and it has been equally difficult to remove all the cortex and leave the medullary tissue.

TABLE II

EFFECT OF BILATERAL NEPHRECTOMY UPON THE OPSONIC ACTIVITY OF THE BLOOD SERUM

Cat No. 3. Black, male. Right kidney removed 1/17/30. Left kidney removed 2/12/30. Cat recovered rapidly. The cat was bled as shown below, and the opsonic activity of the blood serum determined.

	Serum Dilutions (Ratio)					
	Undiluted Serum	1:4	1:16	1:64	1:256	1:1,000
Determination 1						
Cat bled 2/14/30, 2:00 P.M.						
Opsonic activity	75	63	48	35	26	19
Control serum	76	62	50	38	25	17
Spontaneous phagocytosis	15	15	15	15	15	15
Determination 2						
Cat bled 2/16/30, 5:00 P.M.						
Opsonic activity	80	65	51	34	24	16
Control serum	82	67	45	35	28	21
Spontaneous phagocytosis	17	17	17	17	17	17

The cat was killed after being bled on 2/16/30 to obtain tissues for other experiments. Life span, 4 days. At time of death animal was prostrate.

If an active extract of the cortical part of the adrenal gland could be obtained, a way might be opened whereby the relationship between the cortex of the adrenal gland and the maintenance of the resistance might be proved. Such an extract has been obtained by

Swingle and Pfiffner (1930), and it has been shown by them that this extract contains the active cortical principle. When treated with this extract, bilaterally adrenalectomized cats have lived more than 100 days, have gained weight, and have shown normal activity. The life-span of non-treated double-adrenalectomized cats is but 6–8 days.

The effect of this extract upon the opsonic activity of double adrenalectomized cats is now being studied in this laboratory.

The results of these experiments make several facts evident. Cats which have been deprived of the adrenal glands show a marked decrease in the opsonic activity of their blood. In the terminal stages of the adrenal insufficiency the opsonic activity almost disappears. That the decrease is a specific result of adrenal deficiency rather than of deranged metabolism is indicated by the fact that there is no change in the opsonic activity of the blood of double-nephrectomized cats in spite of the similarity of the symptoms associated with nephrectomy to those resulting from complete bilateral adrenalectomy.

SUMMARY

Experiments were performed upon cats to determine if there was any change in the opsonic activity of the blood following the complete removal of the adrenal glands.

Bilateral adrenalectomy in cats results in a marked decrease in the opsonic activity of the blood. The amount of phagocytosis resulting from sensitization with normal serum is about 75–85 per cent, while sensitization with serum drawn from double-adrenalectomized cats gives but 18–30 per cent phagocytosis during the later stages of the adrenal deficiency.

The blood of double-nephrectomized cats shows no change from the normal in opsonic activity.

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AN EXPERIMENTAL STUDY OF THE OPSONINS OF THE BLOOD. II. EFFECT OF IN VITRO IRRADIATION WITH ULTRA-VIOLET AND X-RAY[1]

(One figure)

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IN REVIEWING the literature in connection with the first part of this study, it was noticed that certain factors of the defense mechanism had been studied in relation to the effect of ultraviolet radiation and X-radiation but that other factors, equally important, had been completely ignored in such experiments. The opsonic activity of the blood was one of the phases of bodily defense which had been almost completely overlooked in radiation experiments.

Some work has been performed, *in vivo*, upon the relation of the defense mechanism to radiation. Sonne (1922) found that animals injected with a minimal lethal dose of diphtheria toxin and then treated with the radiation from a carbon-arc lamp for a period of 2 hours withstood the toxin better than did non-rayed control animals receiving the same dose of toxin. He thinks, however, that the toxin may be destroyed by the heating action of the luminous rays which accompany the shorter rays. These longer rays raise the temperature of the blood to above fever temperature; about 48° C. directly under the skin. He believes that more than 40 per cent of the toxin is destroyed, directly, by this heating action.

Koopman (1924), in addition to showing that the complement, in the blood of a guinea pig irradiated for an hour, was almost double, also showed that if the animals were exposed, overly long, to the action of the rays the complement was markedly diminished. An exposure of 3 hours' duration was lethal, and at the time of death no complement could be found in the blood serum. He also showed that if serum was irradiated, *in vitro*, for 3 hours the complement action

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[1] I wish to acknowledge my indebtedness to Professor W. W. Swingle for suggesting this study and for his interest during the course of the work.

was destroyed. Courmant, Nogier, and Dufourt (1913) were among the first to demonstrate this harmful effect of *in vitro* radiation of blood serum upon its complement activity. More recently Gordon and Wormall (1928) have corroborated some of the work of Koopman by showing that the complement action of the serum may be destroyed by exposing the serum to ultra-violet rays. Irradiation of the serum for 2 hours brings about a marked decrease in the complement activity, and an exposure of 3 hours' duration inactivates the complement completely. They also performed *in vivo* experiments. They exposed guinea pigs to ultra-violet rays over a period of several days and found that the exposure had practically no effect upon the activity of the complement and, also, that there was no change in complement activity when the animals were kept in the dark.

One investigator, Albela (1922), found no change in the opsonin content of the blood of guinea pigs which had been exposed to ultra-violet radiation. He obtained similar results with rabbits.

As far as could be determined, no experiments have been performed to determine the effect of *in vitro* radiation of serum upon the opsonic activity of such serum. A series of experiments were, therefore, performed to discover whether it were possible to bring about a change in the opsonin content of normal blood serum by exposing the serum to the action of ultra-violet rays and to X-rays.

MATERIALS AND METHODS

The same technique of opsonic activity determination was used in these experiments as had been used in Part I of this study (Blanchard, 1931). The leucocytes were obtained by intraperitoneal injection of and subsequent withdrawal of about 200 cc. of 0.9 per cent NaCl. The leucocytes were centrifuged slowly, washed, and suspended in a small amount of 0.9 per cent NaCl in the manner reported by Mudd, Lucke, McCutcheon, and Strumia (1929). A suspension of collodion particles, prepared by the method given by Loeb (1922), was used as the antibody. The blood for the experiments was drawn by cardiac puncture and was treated in a manner which will be described.

During this series of experiments two sources of ultra-violet rays were employed. The first was a Westinghouse mercury-arc lamp having a bulb of Corex glass, and the second source was a Hanovia type mercury-arc lamp in a quartz bulb.

The blood serum, after being separated from the cells, was divided in two parts: one portion was used as the experimental serum and the other part was used as the control serum.

In the ultra-violet experiments the serum was exposed to the radiation in an open watch crystal at a distance of 10 inches from the lamp. Accurate observations were made of the temperature changes occurring in the serum during the exposure to the rays; and as regards temperature regulation, the Hanovia lamp was more convenient. During a half-hour's exposure under this lamp the temperature of the serum never rose above 26° C. During a half-hour's exposure under the Westinghouse lamp there occurred a considerable rise in temperature due to the presence of a large amount of the longer rays. Using this lamp, the temperature of the serum would rise as high as 40° C. When this latter lamp was used, the control serum was placed in an incubator for a time equal to the exposure time of the experimental serum, and the incubator was kept at a temperature equal to (in most cases a little higher than) the temperature of the serum being irradiated. This would seem to control the temperature factor in such a way that any changes taking place in the opsonic activity could be ascribed to the action of the ultra-violet rays rather than to any heating effect.

A very similar technique was employed in exposing the serum to X-rays. One portion of the serum was radiated, and the second part was used as the control for the experiment. A Coolidge tube was used as the source of X-rays, and the serum was exposed to the radiation at a distance of 12 inches from the target of the tube. When exposed in this manner, the temperature of the serum never rose above 26° C., which was approximately room temperature. The tube was supplied with 65 kilovolts at 6–7 milliamperes, and this resulted in rays of medium length being emitted by the tube.

Normal cats were used throughout this experiment as the source of blood serum.

RESULTS AND DISCUSSION

Normal serum was exposed to ultra-violet rays and to X-rays for varying times. From this exposed serum and from the control serum serial dilutions were made with 0.9 per cent NaCl, and the opsonic activity of the serum and its dilutions determined in the manner described by the writer in the first section of this study (1931).

TABLE I

EFFECT OF ULTRA-VIOLET RADIATION UPON THE OPSONIC ACTIVITY OF THE BLOOD SERUM

In all the experiments the serum was divided into two portions: one part was radiated, and the other part was used as the control. Serial dilutions of the serum were made.

	SERUM DILUTIONS (RATIO)					
	Undiluted Serum	1:4	1:16	1:64	1:256	1:1,000
Experiment No. 1						
Serum exposed to ultra-violet rays for 10 minutes						
Opsonic activity	43	35	19	17	18	16
Control serum	82	74	61	49	33	21
Spontaneous phagocytosis	17	17	17	17	17	17
Experiment No. 3						
Serum exposed to rays for 15 minutes						
Opsonic activity	38	26	20	14	16	15
Control serum	81	67	45	35	28	17
Spontaneous phagocytosis	16	16	16	16	16	16
Experiment No. 4						
Serum exposed to rays for 20 minutes						
Opsonic activity	22	19	16	14	15	13
Control serum	78	72	48	33	18	15
Spontaneous phagocytosis	14	14	14	14	14	14
Experiment No. 6						
Serum exposed to rays for 30 minutes						
Opsonic activity	18	12	15	11	12	13
Control serum	82	69	53	38	23	17
Spontaneous phagocytosis	15	15	15	15	15	15

The results of several of these experiments are given in Tables I and II.

The results shown in Table I show clearly that when normal blood serum is exposed to the action of ultra-violet rays for a period of $\frac{1}{2}$ hour, the opsonic activity of the serum is almost completely destroyed. Marked decrease in opsonic activity results from exposures of shorter duration. Thus, while the undiluted serum exposed for a time shorter than $\frac{1}{2}$ hour may show some opsonic activity, this activity entirely disappears in dilutions as low as 1:16.

TABLE II

EFFECT OF X-RAYS UPON THE OPSONIC ACTIVITY OF NORMAL BLOOD SERUM

The serum was divided into two portions: one part was radiated, and the other portion used as the control. Serial dilutions of the serum were made.

	Serum Dilutions (Ratio)					
	Undiluted Serum	1:4	1:16	1:64	1:256	1:1,000
Experiment No. 2						
Serum exposed to X-rays for 15 minutes						
Opsonic activity	42	35	27	19	15	12
Control serum	82	68	52	39	24	17
Spontaneous phagocytosis	14	14	14	14	14	14
Experiment No. 3						
Serum exposed to rays for 20 minutes						
Opsonic activity	35	27	16	12	14	11
Control serum (same as in Experiment 2)	82	68	52	39	24	17
Spontaneous phagocytosis	14	14	14	14	14	14
Experiment No. 4						
Serum exposed to rays for 30 minutes						
Opsonic activity	20	17	11	9	10	12
Control serum	75	60	47	32	20	13
Spontaneous phagocytosis	12	12	12	12	12	12
Experiment No. 6						
Serum exposed to rays for 45 minutes						
Opsonic activity	18	16	15	17	14	15
Control serum	76	64	49	35	23	16
Spontaneous phagocytosis	15	15	15	15	15	15

Very similar results were obtained when the serum was exposed to the action of X-rays, as can be seen in Table II.

The foregoing results clearly indicate that the opsonin of the blood serum is completely inactivated by a 45 minutes' exposure to medium length X-rays. Exposures of shorter duration result in marked decrease in the opsonic activity.

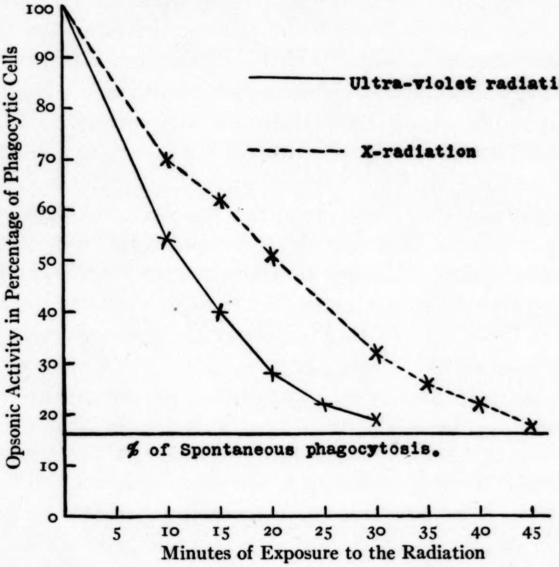

FIG. 1.—Changes in opsonic activity resulting from *in vitro* radiation of normal blood serum.

The changes taking place in the opsonic activity of the blood serum following exposure of the serum to radiation can be shown graphically, as in Figure 1. In plotting this curve, the opsonic activity, in terms of percentage of phagocytic cells, is plotted against the time of exposure of the serum to the radiation. All of the experiments have been grouped together, and the data have been corrected on the basis of normal activity resulting in 100 per cent phagocytosis.

The two curves showing the change in opsonic activity following exposure of the serum to ultra-violet and to X-rays are very similar in shape, differing however in slope. No attempt has been made to analyze these curves mathematically.

The means by which radiation brings about destruction of the opsonic activity of the serum cannot be stated with absolute assurance; but a possible explanation, based upon and agreeing with the experimental results, can be given.

No one has been able to determine the chemical nature or composition of opsonin. It is the belief of many investigators that opsonin is very similar in chemical nature to the complement; and this latter substance is, possibly, protein-like in nature.

Gordon and Wormall (1928) state that many investigations as to the action of irradiation upon biological fluids seem to point to some effect of the radiation upon the proteins present in such fluids, and it is very probable that the action of the rays is due to changes in the state of the colloids following the radiation. This work of Gordon and Wormall (1928) indicates that the complement is protein-like in nature and that the injurious effect brought about by radiation is the same as the effect resulting from heating the complement, namely, coagulation of the proteins present. The heat sensitivity of opsonin is identical with that of complement; the opsonin is inactivated by heating the serum for 1 hour at a temperature of 55°–60° C. This would indicate a similarity, as far as chemical nature and colloidal state is concerned, between opsonin and complement. Thus, it is probable that irradiation of the serum with ultra-violet or X-rays inactivates the opsonin, possibly a protein-like substance, by effecting a change in the state of the colloids of the opsonin.

Experiments in which radiation plays a part could be greatly facilitated, and the results obtained by various workers could be correlated with greater accuracy and assurance, if some method could be devised for accurately measuring the quantity and quality of rays being given off by ultra-violet lamps or by X-ray tubes.

SUMMARY

Experiments were performed to determine the changes in opsonic activity which would follow *in vitro* radiation of normal blood serum. Cat serum was used in the experiment.

Irradiating normal blood serum with ultra-violet rays for a period of ½ hour or with X-rays for 45 minutes results in a marked decrease in the opsonic activity of the blood serum. The temperature factor was controlled at all times.

A possible explanation for this decrease in opsonic activity following radiation of the serum is given in the text.

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STUDIES IN WATER METABOLISM OF THE CAT. THE INFLUENCE OF DEHYDRATION ON BLOOD CONCENTRATION, THERMOREGULATION, RESPIRATORY EXCHANGE, AND METABOLIC-WATER PRODUCTION[1]

(Five figures)

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INTRODUCTION

THE author (1925) showed that, in a number of poikilothermic animals representing several phyla and xeric, mesic, and hydric habitats, dehydration resulted in an increased carbon-dioxide production. This suggested that, in animals which exist under arid conditions, where water is one of the principal factors in survival, a decrease in water content of the body might lead to a greater production of water of oxidation through increased respiratory exchange.

Before the effects of dehydration could be studied in the homotherms, it became necessary to know whether the existence of a connection between the metabolism of water and the homothermal mechanism, as proposed by Balcar, Sansum, and Woodyatt (1919), Barbour and Tolstoi (1922), and Rogers (1924*b*), would manifest itself by an increase in body temperature when the animals were dehydrated by water deprivation in a manner somewhat simulating desert conditions. Balcar, Sansum, and Woodyatt (1919) dehydrated dogs by intravenous injections of diuretic agents, glucose, lactose, and sodium-chloride solutions, and reported an increase in body temperature of 11°–23.6° F. Keith (1924) repeated the experiments and found that with smaller dosage but a higher ratio of water output there was no increase in body temperature if the loss of water did not exceed 10 per cent of the body weight. With greater losses the maximum increase in body temperature was less than 4° F., and

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[1] The author takes pleasure in his acknowledgment of the helpful suggestions and criticisms of Dr. W. C. Allee, under whose supervision this work has been accomplished.

in many experiments less than 1° F. Barbour and Tolstoi (1922) exposed dogs to hot and cold baths and observed respectively concentration and dilution of the blood. These responses could not be elicited in the absence of the heat-regulating mechanism, brought about by the transection of the cord at the sixth cervical level.

Since the work reported in this paper has been in progress, dehydration experiments with sugar and salt solutions have been repeated by many workers with conflicting results. Keith and Whalen (1926) reported that marked hyperpyrexia did not occur when all possible toxic factors connected with preparation and administration of the sugar solutions were excluded. Andrews (1926) showed that if a dog was allowed to ventilate his tongue freely he could regulate his body temperature "fairly well" in extreme dehydration from intravenous injections of sugar and salt solutions; while, if its mouth was tied shut, there was a rise of 1°–2° F. with moderate dehydration and in extreme dehydration a terminal rise of 10°–12° F. This conclusion is substantiated by the fact that dogs depend on the upper respiratory tract mainly for water evaporation and by the statement of Roundtree (1922) that the rectal temperature of tracheotomized dogs rose from 39° to 42° and 44° C. when they were placed in the sunshine. This source of error seems to have been overlooked by other workers, and may have been responsible for conflicting reports on the effects of sugar diuresis on body temperature.

The literature on water metabolism is very extensive and has been reviewed in part by Roundtree (1922) in relation to water balance, by Marriott (1923) in relation to blood changes observed by pediatricians in the acute anhydremia of infants, by Schiff (1929) in relation to infant toxicosis following water losses; and recently some of the literature relative to water balance has been reviewed by Adolph (1930). Underhill and co-workers (1923) reported blood concentration changes in extensive superficial burns.

Animal experimentation on the general physiological effects of dehydration has been rather limited and with little uniformity in conditions. Czerny (1894) deprived young cats of food and water and studied body-temperature and blood-concentration effects when the animals were kept in dry air at temperatures from 31° to 39.5° C. Straub (1899) deprived young dogs of water for three days and re-

reported on metabolism and circulation determinations. Adolph (1921) studied the effects of water loss in man over short periods of time. Underhill and Kapsinow (1922) reported on blood concentration in fasting rabbits deprived of water, and Underhill and Roth (1922) found an increase in hemoglobin in fasting dogs deprived of water. Keith (1922) reported a decrease in blood volume of dogs when food and water were withheld. Korth and Marx (1928) found fluctuations in hemoglobin in the blood of dogs during thirst.

The original purpose of these experiments was to determine the effects of disturbance of the water balance on the ability of the animal to regulate body temperature. The limited knowledge available on the effects of dehydration, as pointed out by Roundtree (1922) and more recently by Gamble (1929) and Schiff (1929), seemed to justify the extension of the problem to include blood counts as an indication of the extent of anhydremia caused by extreme dehydration, to attempt the estimation of metabolic water production, and to determine the effect of dehydration on the respiratory exchange.

It should be emphasized that, unlike the majority of experiments reported on dehydration effects, the animals in these experiments were fed regularly. Starvation undoubtedly modified the results in some cases near the end of the experiments, but usually the latter were stopped soon after the animal refused food on account of the mechanical difficulties encountered in deglutition from reduced salivary secretion. This does not necessarily mean that the animals ate sufficient food to supply the total energy output, and without doubt the effects of dehydration are obscured at times by starvation.

A method has been devised which produced not so rapid but just as severe dehydration, without toxic etiologic factors which might accompany intravenous injections of hypertonic solutions, which permitted of determinations of water losses by evaporation as well as through other sources and which provided for air-sampling for gas analyses. These features have not been incorporated generally in other methods.

EXPERIMENTAL PROCEDURE

The dehydration apparatus (Fig. 1) consisted of a 22-liter bell jar with ground flange, which rested on plate glass and served as the animal chamber. Two porcelain pans of a diameter slightly less than that of the bell jar were fitted into one another. The bottom of the inner pan was perforated and supplied with feet which rested on the inner bottom of the outer pan. These were placed in the animal chamber and prevented the cat from consuming any appreciable quantity of its urine. The neck of the jar was provided with an inlet and outlet tube, a glass tube with stopcock through which samples of air could be drawn, a thermometer, and a mercury manometer which facilitated tests for leaks in the system. Outdoor air was drawn through two 1-quart William's gas-washing bottles containing 500 cc. of concentrated H_2SO_4 c.p., through an empty bottle which served as a trap, another bottle containing granulated zinc, a third bottle filled with glass wool, and then into an absorption tube containing P_2O_5. The air then passed through a flowmeter and into the animal chamber. From the animal chamber the air was drawn into a 1-liter volumetric flask which contained 600 cc. of H_2SO_4. This flask was fitted with a glass intake tube through which the air passed into the acid through fine holes which broke up the air bubbles so that it was not necessary to use two H_2SO_4 ab-

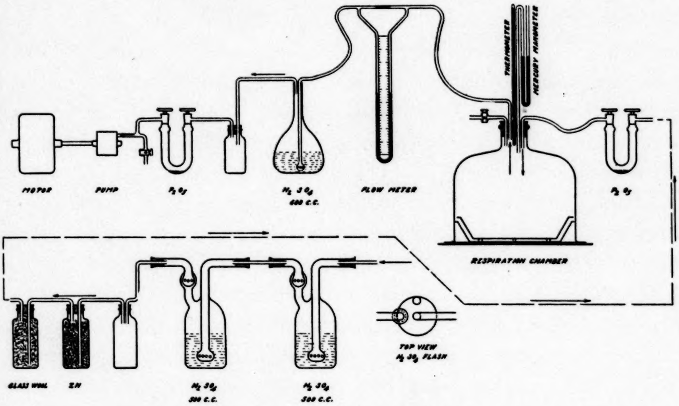

FIG. 1.—Apparatus used in dehydration of cats and collection of water vapor from respiration and evaporation from excreta. Bottles not labeled are traps. The apparatus was connected with an outdoor air intake.

sorption flasks to absorb the water of respiration and evaporation. This absorption flask was connected by glass tubing, without rubber, to a small empty bottle for a trap, which was always weighed with the flask. At this point another absorption tube containing P_2O_5 was inserted in the system. When the P_2O_5 showed the presence of moisture, the acid in the absorption flask was renewed. The same method was used for the William's gas-washing bottles. From the P_2O_5 tube the air passed into the pump, which was driven by an electric motor. A valve near the pump made it possible to keep the flow of air through the animal chamber constant. As the quantity of acid in the flask increased due to water absorption, the resistance in the system increased and the air flow decreased. This was overcome by the selection of absorption tubes with small bore inlet and outlet apertures for the P_2O_5. These provided a fixed resistance in the air line, which was always higher than the total resistance elsewhere in the system even with maximum dilution of the H_2SO_4 from water absorption. At intervals, air, at the usual rate of flow, was bubbled through methyl-orange solution to test for H_2SO_4. These tests were consistently negative. The flowmeter was calibrated on a standard meter tester.[1] The apparatus could be quickly tested for leaks by the adjustment of the valve to create a considerable vacuum, followed by closure of the two ends of the system and subsequent observation of the mercury in the manometer of the animal chamber.

Body-temperature determinations were made by way of the rectum with a standard clinical thermometer. Blood counts were made with a standard haemacytometer. For red blood corpuscles, the blood was diluted 1–200 with Toisson's solution. One per cent acetic acid and a dilution of 1–20 were employed for white counts. Usually, 80 squares were counted for red corpuscles and 16 large squares for the white corpuscles.

Gas analyses were made in a Newcomer modification of the Haldane gas-analysis apparatus. The procedure described by Carpenter (1915) was followed throughout. Gas samples were drawn by means of a mercury pump directly from the animal chamber into gas-collecting tubes of 125 cc. capacity provided with stopcocks at either end. Ten cubic centimeters of acidulated water in the collecting tubes served to completely saturate the air with moisture before the sample was introduced into the gas-analysis apparatus. As the capacity of the burette of the Haldane was only 10 cc., a 75-cc. sample was sufficient for several analyses.

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[1] The author is indebted to the Tucson Gas, Electric Light and Power Company for the use of apparatus and assistance in the calibration of the flowmeters.

The experiments were conducted in Tucson, Arizona, upon full-grown cats acclimated to the semi-arid conditions of that region. Blood counts were made on many cats in the attempt to select healthy individuals. Before actual experimentation was begun, each of the experimental animals had been for some time under observation in the laboratory, on a diet of cooked, lean meat; they were accustomed to handling and to confinement. The experiments on 13 males and 3 females and the temperature control data on 6 cats have been included in this report. The females had been isolated sufficiently long to determine that they were not pregnant. Data have not been included from several experiments which were discontinued because of mechanical difficulties with apparatus or illness of the animal from diarrhea which, since it occurred early in the experiment, could be attributed to chance infection rather than a lowered resistance from dehydration.

For the experiments in which carbon-dioxide production and oxygen absorption were not determined, the procedure was the same throughout. Blood samples were taken, the rectal temperature recorded, the animal weighed, the desiccator for absorption of water from the animal chamber weighed, and the animal placed in the apparatus. The valve was adjusted to give the desired flow of air, and the animal was undisturbed until the next observations. At 12-hour intervals the animal was removed from the desiccator, weighed, rectal temperature recorded, and fed as much cooked, lean meat as it would eat. All visible fat was removed from the meat, which almost without exception was beef. A 10-gm. sample of the food was taken for determination of water content by drying to constant weight at a temperature of 100° C. The temperature of the animal chamber was recorded immediately before the animal was removed. The urine and feces were collected from pans and weighed. The water content of the feces was determined in the same manner as for the food sample. The outlet desiccator was weighed and water losses from evaporation recorded. The weight of the food eaten was determined and the cat weighed to check any loss of excreta while it was out of the animal chamber and as a check against the weight of food consumed. At 72-hour intervals blood samples were taken and red and white corpuscle counts made. With the exception of cat No. 15, the animals received no water throughout the experiment except that from the cooked, lean meat; cat No. 15 received no free water but for 5 of the 16 days was fed uncooked, lean beef which contained a higher water content than the cooked meat.

In the experiments in which respiratory exchange was determined, the procedure was identical with the foregoing except that the animals were kept in the animal chamber for several days prior to the experimental period to accustom them to all the experimental conditions except dehydration. For this period, outdoor air with normal degree of humidity was passed through the animal chamber at the same rate as when the animal was to be desiccated. For this adaptation period, 2–4 days, gas analyses were made daily and the cats were fed lean, cooked meat and given water *ad libitum*. Usually, after 24 hours in the apparatus, the excitement from the close confinement had subsided and the rate of respiration was normal. These preliminary analyses furnished the data for comparison of normal and experimental carbon-dioxide production and oxygen consumption. For the duration of the experiment gas analyses of the air from the animal chamber were made at 72-hour intervals. Barometer readings were taken at the time of analysis. The gas samples were taken when the animal was at rest and from 10 to 12 hours, occasionally less, after the last meal.

GENERAL OBSERVATIONS

The cats apparently suffered more from thirst during the first 5 or 6 days of the experiment and, for this period, were more irritable than later. After this period there was a decided reduction in response to external stimuli, particularly noted when punctures were made to obtain blood samples. As dehydration progressed, the cats slept more and, when not asleep, exhibited marked somnolence. In some of the longer experiments there was almost a total absence of response to skin stimuli at the close of the experiment. The skin became rough and loose, with marked reduction in turgor and increased hair loss. The feces were hard and dry. Diarrhea was observed in only one case. Kudo (1921*a*) observed a greater tendency toward diarrhea in his studies on the effect of thirst on weight of rats. ✓

Opacity of the lens, extension of the penis, hemorrhage from the nose, and paralysis of the posterior extremity, all observed by Kudo, were not present in the cats. Congestion of the conjunctiva was observed in some cases, as found by Kudo in rats. In extreme dehydration there was a marked depression of the central nervous system, accompanied by aphonia and dysphagia. There seemed to be little tolerance of thirst.

EXTENT OF DEHYDRATION

The administration of diuretics and the intravenous injections of hypertonic solutions to reduce the water content of the body involve radical changes in the organs which have to do with the regulation of water balance. The experiments reported here were designed to diminish the water content of the animal gradually to afford the animal an opportunity to adjust its needs, so far as possible, to a diminution in total water content uncomplicated by the action of drugs or other foreign substances.

Unlike the majority of work on dehydration, a careful determination of all water leaving the body has been made, as well as all water received by the animal. The intake-output ratios given below (Table I) include water lost by respiration, by evaporation from the body surface, and from the excreta. It has not been possible to distinguish between water of respiration and other water losses, because evaporation from urine and feces, when present in the pans, added to the weight of the outlet desiccator.

While the intake-output ratios are important, the actual difference between the total water intake and output expressed in percentage of the original body weight, and designated as negative water balance, seems to offer a better basis for some comparisons. The daily output of urine at the expense of the body tissue is surprisingly high in severe dehydration (Table I). The low intake-output ratio for cat No. 15 was the result of the large quantity of uncooked meat which it ate for 5 of the 16 days of the experiment. This cat weighed only 1,225 gm. at the beginning of the experiment and gained in weight when fed uncooked, lean meat. This would indicate that carnivorous animals may live long periods without access to free water. Under the conditions of the experiment, from 12 to 17 days was the maximum limit of desiccation without death intervening. The quantity of food taken and variations in room temperature were important factors in the duration of desiccation. Hall (1922), under somewhat similar conditions, found that the house mouse withstood desiccation for 8–16 days, while the wood mouse and meadow mouse gave up water more quickly.

As one phase of the problem was a study of the ability of the animal to regulate body temperature with different degrees of dehydration, no attempt was made to keep the animal chamber at constant temperature. The greatest variation recorded in the temperature of the animal chamber for the duration of a given experiment was 7° C. One-half of the experiments had recorded temperature changes of less than 5° C. These variations were principally differences between morning and evening temperatures of the room.

Czerny (1894) found that young cats, 209–685 gm., died after 15 days without food and water when kept at a temperature of 31°–32.5° C.; after 8 days at a temperature of 33.1–35.0° C., and after 20 hours at a temperature of 38.8–39.5° C. Such temperatures are much higher than the animal-chamber temperatures recorded in these experiments. Rogers (1924*a*) showed that complete destruction of the thalamus in decerebrate pigeons is followed by excessive diuresis with estimated water loss at the rate of 10–60 per cent of fasting weight in 24 hours. With water loss at a rate greater than 18 per cent of body weight in 24 hours, the birds could not be kept alive for periods of more than 36 hours. Czerny (1894) observed that young cats at a temperature of 33°–35° C. lost 45.9 per cent of body weight, while young cats at 38.0°–39.0° C. died when the body weight loss averaged 24 per cent.

THERMOREGULATION DURING DEHYDRATION

Since the literature on heat regulation has been reviewed by Barbour (1921), only the papers having a significant relation to water

TABLE I

Daily intake and output of water, with ratios and negative balance in percentage of original body weight

Days	Cat No. 1		Cat No. 5		Cat No. 15		Cat No. 17	
	Intake	Output	Intake	Output	Intake	Output	Intake	Output
1	28.2	123.0	45.5	124.0	18.7	52.0	19.3	57.0
2	49.9	118.0	51.3	142.6	75.9	60.2	116.3	119.4
3	89.2	106.0	70.4	120.9	91.3	79.5	53.2	110.9
4	57.3	139.0	31.8	238.8	95.8	78.0	17.1	187.0
5	79.9	127.1	70.0	59.7	71.7	88.5	14.1	95.5
6	45.5	115.5	20.4	109.6	17.3	85.9	15.7	71.0
7	24.5	91.0	40.0	121.3	99.5	69.0	1.9	59.5
8	49.3	99.4	17.6	63.1	87.6	98.0	......	34.0*
9	15.3	110.5	26.5	78.2	24.7	80.8	......	......
10	43.7	74.0	25.9	71.3	19.9	59.0	......	......
11	118.4	94.7	38.5	73.4	60.4	51.0	......	......
12	6.8	106.5	27.9	82.6	32.0	65.5	......	......
13	9.4	69.0	22.7	53.4	24.1	57.0	......	......
14	......	51.0*	11.3	113.9	28.0	44.0	......	......
15	......	......	......	......	11.7	60.0	......	......
16	......	......	......	......	4.3	42.8	......	......
Totals	617.4	1,424.7	499.8	1,452.8	762.9	1,071.2	237.6	734.3
Ratios	1:2.30		1:2.90		1:1.40		1:3.09	
Negative balance	28.4%		30.9%		25.1%		22.7%	

* =12 hours only.

TABLE I—*Continued*

Days	Cat No. 20		Cat No. 22		Cat No. 23		Cat No. 30	
	Intake	Output	Intake	Output	Intake	Output	Intake	Output
1	0.0	49.5	21.8	49.5	29.0	71.0	50.8	92.0
2	30.1	33.5	30.9	138.0	24.0	72.0	84.0	110.9
3	12.5	59.0	26.4	113.4	25.0	93.5	95.0	117.0
4	19.9	209.0	54.9	99.5	12.4	82.1	67.4	106.0
5	27.2	64.0	48.6	131.5	19.4	55.5	57.6	107.5
6	22.2	82.9	26.1	142.0	13.3	58.5	83.8	105.8
7	30.6	30.0	41.6	111.5	8.9	46.5	53.6	105.0
8	18.7	131.5	21.4	93.5	17.2	47.0	11.8	103.2
9	15.2	66.0	26.4	71.0	25.5	29.7	20.9	100.4
10	61.7	73.5	0.6	76.0	26.2	59.1	13.0	65.4
11	20.5	46.5	6.1	32.0	3.3	62.5	22.8	58.8
12	......	54.0*	19.5	75.4	......	......	10.8	72.3
13	......	......	28.2	55.5	......	......	......	......
14	......	......	......	31.0*	......	......	......	......
Totals	258.6	899.4	352.5	1,219.8	204.2	677.4	571.5	1,144.3
Ratios	1:3.47		1:3.46		1:3.31		1:2.00	
Negative balance	32.9%		26.3%		33.9%		20.9%	

* =12 hours only.

TABLE I—*Continued*

Days	Cat No. 33		Cat No. 35		Cat No. 36		Cat No. 48	
	Intake	Output	Intake	Output	Intake	Output	Intake	Output
1	69.6	199.5	50.9	164.7	20.2	102.5	13.4	70.5
2	64.3	102.5	70.5	119.5	45.1	107.0	10.3	47.0
3	44.9	135.5	66.1	140.0	40.3	70.5	10.5	46.0
4	57.2	99.2	51.3	56.5	22.5	105.0	0.0	58.2
5	55.8	117.0	34.0	103.4	45.9	74.9	1.4	37.5
6	35.7	123.5	24.0	115.0	31.7	90.0	5.1	41.5
7	31.7	61.5	18.1	106.5	24.1	56.0	0.8	39.5
8	19.7	81.0	25.3	92.5	18.5	76.0	0.8	42.5
9	25.7	68.0	23.8	88.5	19.0	71.1	....	46.5
10	16.5	62.0	24.1	61.0	17.9	57.5	....	....
11	14.8	63.5	20.1	67.5	16.2	74.0	....	....
12	23.8	55.5	4.7	56.5	9.9	60.5	....	....
13	16.6	59.5	....	....	11.7	61.5	....	....
14	7.4	56.5	....	....	5.9	56.0	....	....
15	....	....	....	....	3.0	44.0	....	....
Totals	483.7	1,284.7	412.9	1,171.6	331.9	1,106.5	42.3	429.2
Ratios	1:2.65		1:2.83		1:3.33		1:10.1	
Negative balance	24.2%		31.6%		28.3%		16.6%	

TABLE I—*Continued*

Days	Cat No. 50		Cat No. 54		Cat No. 56		Cat No. 58	
	Intake	Output	Intake	Output	Intake	Output	Intake	Output
1	12.1	38.5	38.0	70.5	8.9	108.2	22.2	77.0
2	28.9	93.5	50.0	149.0	10.6	68.0	18.5	81.5
3	29.2	64.7	15.6	106.6	1.7	66.4	8.6	64.0
4	15.9	79.5	24.1	76.5	0.0	53.5	3.3	45.5
5	21.0	63.5	30.7	76.0	....	46.3	0.3	60.7
6	28.6	86.0	16.9	87.6	....	42.0	0.9	43.5
7	23.4	74.0	10.9	64.0	....	....	0.0	40.0
8	16.4	63.0	0.0	67.9	....	....	....	48.0
9	12.5	57.0	....	42.5	....	....	....	45.0
10	16.0	54.5	....	49.4	....	....	....	....
11	14.9	50.0	....	40.0	....	....	....	....
12	6.4	67.4	....	38.5	....	....	....	....
Totals	225.3	791.6	186.2	868.5	21.2	384.4	53.8	505.2
Ratios	1:3.51		1:4.66		1:18.1		1:9.39	
Negative balance	29.0%		30.1%		20.2%		19.3%	

metabolism, and especially dehydration, will receive attention here.

Czerny (1894) reported an increase in body temperature of young cats deprived of water and food at temperatures of 33°–39° C., but did not state extent of increase. Sonderstrom and DuBois (1917) found a diminished output of water in typhoid-fever patients. Meyer, Cohen, and Carlson (1918) produced fevers experimentally in dogs and recorded a diminution in gastric secretion. Balcar, Sansum, and Woodyatt (1919) produced body temperature increases of 11°–23.6° F. in dogs dehydrated by intravenous injections of sugar and salts, and concluded that fever is due to a deficit of free water resulting from an abnormal tendency on the part of colloids to bind water. Barbour and Howard (1920) found a concentration of the blood in coli fever in dogs, and Barbour and Freedman (1920) found that salivary secretion following pilocarpine was much reduced in coli-fever dogs. Keith (1924) dehydrated dogs by intravenous injections of glucose and reported an increase in body temperature, but repeated the work later (Keith and Whalen, 1926) and found that there was no marked hyperthermia when toxic factors were eliminated.

Rogers (1924*a*) produced hyperthermia in decerebrate pigeons by complete destruction of the thalamus with electrocautery. Hyperthermia only occurred when there was a diuresis sufficient to produce anhydremia. Rogers suggested that the rate of water loss was more important in production of hyperthermia than the actual quantity lost. Kayser (1929) found that decerebrate pigeons with cauterized thalamus became polyuric and were not able to regulate temperature, but that when the diuresis ceased, the ability to regulate temperature reappeared immediately. Kayser also produced polyuria in rabbits by puncture of the fourth ventricle and recorded a hypothermia accompanied by a reduction of 14.2–27.6 per cent in respiratory exchanges and concluded that thermoregulation fails when hydration equilibrium is disturbed. Dragstedt and Ellis (1929) dehydrated dogs to depress the volume of gastric juice secreted, and reported normal body temperature throughout the dehydration periods. This brief and incomplete summary of the literature on the relation of thermoregulation and water metabolism seems to indicate some connection other than the passive rôle of water in heat conservation and dissipation for which it is so admirably suited because of its peculiar physical properties.

The results of these experiments (Tables II and III) clearly show

TABLE II

AVERAGES OF MORNING AND EVENING TEMPERATURES OF DEHYDRATED CATS AND CORRESPONDING TEMPERATURES OF THE RESPIRATION CHAMBER IN DEGREES CENTIGRADE

Days	Cat No. 1		Cat No. 5		Cat No. 15		Cat No. 17		Cat No. 20		Cat No. 22	
	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber
1	38.8	21.0	39.3	24.7	38.6	22.0	38.5	27.5	38.6	24.7	38.6	22.5
2	38.3	22.1	38.8	24.7	38.3	22.7	38.5	27.3	38.4	25.7	38.7	24.0
3	38.1	22.5	38.6	24.2	38.7	21.7	38.3	25.5	38.3	25.7	38.2	24.2
4	38.3	22.5	38.5	23.2	39.1	23.8	38.3	26.0	38.5	26.3	38.7	25.3
5	38.1	22.5	38.4	24.0	38.7	24.0	38.2	26.2	38.4	26.0	38.6	24.5
6	37.5	20.0	38.6	25.0	38.5	25.2	38.3	25.7	38.4	24.7	39.3	25.7
7	37.6	23.5	38.2	24.5	38.4	26.0	38.3	24.5	38.6	21.5	38.7	23.6
8	38.3	24.0	38.2	25.5	38.9	24.0	38.3	25.5	38.7	23.3	38.4	24.5
9	38.3	23.5	38.1	24.7	38.8	23.0	.....	.....	38.7	24.0	38.6	25.8
10	38.3	24.0	38.1	24.2	38.6	23.5	.....	.....	38.6	25.3	38.5	26.0
11	38.2	23.0	38.0	23.5	38.5	21.7	.....	.....	38.7	23.0	38.4	22.2
12	38.3	24.0	38.4	24.5	38.6	24.2	.....	.....	38.6	24.3	38.4	25.0
13	38.3	24.3	38.6	24.5	38.3	23.5	.....	.....	.....	.....	38.1	20.2
14	.....	.....	38.4	25.0	38.3	25.5	.....	.....	.....	.....	38.5	21.8
15	.....	.....	.....	.....	38.3	24.8	.....	.....	.....	.....	.....	.....
16	.....	.....	.....	.....	38.5	25.7	.....	.....	.....	.....	.....	.....

TABLE II—*Continued*

Days	Cat No. 23		Cat No. 30		Cat No. 33		Cat No. 35		Cat No. 36	
	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber
1	38.4	27.0	37.9	24.2	38.7	27.0	38.7	28.7	38.5	26.4
2	38.7	27.0	38.1	23.8	38.3	24.9	38.5	28.2	39.0	27.5
3	39.2	28.0	38.1	24.7	38.2	25.0	38.1	25.2	38.5	25.6
4	39.1	26.5	38.3	26.5	38.2	25.0	37.7	26.0	38.9	27.4
5	38.7	26.5	38.4	28.1	38.4	26.4	38.3	26.4	38.9	27.0
6	38.5	24.3	38.1	27.2	38.6	27.1	38.4	26.1	38.5	25.5
7	38.6	26.0	38.3	27.0	38.6	27.0	38.4	26.1	38.5	25.6
8	38.7	24.0	38.2	26.6	38.4	27.9	38.7	27.9	38.8	25.5
9	38.6	25.5	38.0	26.1	38.4	26.9	38.4	26.3	38.4	25.7
10	38.5	25.5	38.1	26.9	38.2	26.7	38.2	24.0	38.3	25.2
11	38.2	24.2	38.5	28.4	38.3	26.2	38.1	26.0	38.7	26.5
12	.....	.....	38.5	26.8	38.2	25.7	38.1	25.1	38.9	28.0
13	.....	.....	.....	.....	38.3	27.3	.....	.....	38.6	24.3
14	.....	.....	.....	.....	38.1	25.5	.....	.....	38.9	26.0
15	.....	.....	.....	.....	.....	.....	.....	.....	38.7	25.5

TABLE II—*Continued*

DAYS	CAT NO. 48		CAT NO. 50		CAT NO. 54		CAT NO. 56		CAT NO. 58	
	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber	Cat	Chamber
1	38.4	24.7	38.7	27.2	38.8	27.2	38.6	27.2	38.7	28.0
2	38.4	23.5	38.8	26.7	38.8	26.0	38.5	26.7	38.3	27.7
3	37.8	22.7	39.0	28.7	38.8	28.0	38.8	29.7	38.8	30.0
4	38.1	24.0	38.8	27.5	38.9	29.0	38.9	30.0	39.1	30.5
5	38.1	24.0	38.7	27.0	39.0	29.0	38.6	28.5	38.8	29.5
6	38.3	24.2	38.8	27.7	39.0	30.5	38.6	28.5	38.7	28.5
7	38.3	24.0	38.6	27.0	38.9	30.0	......	......	39.4	28.0
8	38.2	25.5	38.3	25.2	38.6	27.5	......	......	38.8	29.0
9	38.3	26.5	38.3	25.7	38.7	27.5	......	......	38.6	29.5
10	......	......	38.6	26.5	38.9	29.5	......	......	......	......
11	......	......	38.5	27.0	38.9	30.0	......	......	......	......
12	......	......	38.1	25.7	38.6	28.7	......	......	......	......

TABLE III

AVERAGES OF MORNING AND EVENING TEMPERATURES OF CONTROL CATS AND CORRESPONDING ROOM TEMPERATURES IN DEGREES CENTIGRADE

DAYS	CAT NO. 55	CAT NO. 57	CAT NO. 59	CAT NO. 61	CAT NO. 63	ROOM TEMPERATURE	CAT NO. 67	
							Cat	Room Temperature
1	39.0	39.0	39.1	39.1	39.4	23.2	38.9	21.7
2	38.5	38.5	38.8	39.2	39.4	21.5	38.4	20.5
3	38.9	39.4	39.1	39.0	39.5	21.7	38.7	20.5
4	38.5	38.7	38.9	38.3	39.0	21.5	38.7	17.0
5	38.4	38.5	39.0	38.5	40.0	21.0	38.7	21.0
6	38.9	39.2	38.9	38.9	39.0	21.5	38.6	22.0
7	38.7	38.7	38.8	39.0	38.3	21.0	39.0	19.5
8	38.9	38.6	38.7	39.3	38.4	23.0	38.7	21.0
9	38.6	38.6	38.5	39.1	38.3	22.0	38.9	21.5
10	38.2	38.2	38.4	38.5	38.6	22.0	39.0	19.5
11	38.3	38.3	38.4	38.5	38.5	22.2	38.5	21.0
12	38.6	38.6	38.9	38.6	38.9	22.5	38.6	22.2
13	38.8	38.8	38.5	39.1	39.0	22.7	38.5	22.5
14	38.2	38.1	39.2	39.1	38.6	22.0	38.5	21.5
15	38.5	38.5	39.3	38.6	38.5	23.5	....	....

that it is possible to reduce the water content of adult cats practically to the vital limit without disturbance of the thermoregulation mechanism. Within a range of approximately 10° C. of external temperature, it is certain that the body-temperature variations of the experimental animals did not exceed those of the control animals.

The average of morning and evening temperatures tends to obscure the diurnal variations but was necessary to reduce the bulk of data. Whether these animals could have regulated their body temperature, as well as normal cats, when exposed to extremes of external temperature remains to be determined.

BLOOD CONCENTRATION DURING DEHYDRATION

Any disturbance of water metabolism which results in a negative water balance is followed by a concentration of the blood solids. A sudden loss of water from the body results in a greater blood concentration than when the same amount of water is lost over a longer period of time. Adolph (1921) found a 15 per cent concentration of the blood in man with a 5 per cent loss of body weight when the subject was sweated for a short time in a hot bath, as compared to a 2 per cent blood concentration when the same amount of fluid was lost by sweating over a period of 3 hours. Keith (1924) reported concentrations of blood plasma of 24–44 per cent in dogs dehydrated rapidly by intravenous injections of sugar solutions. Skelton (1927) determined that in the cat the skin contained 20 per cent of the total water, the muscles 50 per cent, and the blood 7 per cent. When dehydration takes place slowly, the reserves are drawn upon to restore the blood to a normal condition and blood concentration more nearly represents the condition of dehydration throughout the entire body.

In mammals, a sufficient amount of water must be excreted to hold in solution urea, salts, and other waste products in proper dilution for secretion by the kidneys. Adolph (1922) has demonstrated that there is a maximum concentration of urea and sodium chloride which can be excreted by a given individual. Regardless of this constant demand upon the body for water to eliminate the end-products of metabolism and salts, it has been repeatedly demonstrated that the concentration of the blood may be greatly increased without loss of life. Czerny (1894) reported an increase of 44 per cent in the red corpuscles of young cats desiccated at a temperature of 33°–35° C. Underhill and Kapsinow (1922) reported an increase of 40 per cent in hemoglobin of fasting dogs deprived of water, and Underhill and co-workers (1923) found an increase in hemoglobin of more than 100 per cent in man following extensive superficial burns. Schiff, Eliasberg, and Bayer (1924) reduced the water intake of young children for 3 days and reported an increase of 40 per cent in protein concentration of the blood; and Schiff (1929) reported a 38 per cent increase in blood proteins of young dogs fed milk powder.

A number of methods have been used for the determination of blood concentration: total solids by dry weight, which requires the loss of considerable blood; estimation of hemoglobin by various methods, which cannot be corrected for reduction of hemoglobin without reduction of red corpuscles (Korth and Marx, 1928); determination of total solids with the haematokrit, which fails to record changes in size of corpuscles with various tensions of CO_2 (Smirk, 1928); specific gravity of the blood, an indirect method; the refractometric determination of total proteins; and the increase in the number of erythrocytes. In the present experiments, the latter method was used because the red blood corpuscles are the most stable elements of the blood under the conditions of the experiment. Blood samples were taken from the saphenous vein, without anesthesia, to avoid the concentration of corpuscles in the capillaries of the skin which Utheim (1920) has shown may occur in dehydration.

The effects of dehydration on the blood concentration of adult cats deprived of all water except that contained in cooked, lean meat, as indicated by red corpuscle counts at 72-hour intervals, are shown in Figure 2. A rather wide variation in the normal number of red corpuscles per cubic millimeter of blood may be noted. Two factors may have influenced this: age of the individual and the effect of emotion on the contraction of the spleen. Czerny (1894) gave an average of 7,372,444 corpuscles per cubic millimeter for 3 young cats of 355–416 gm. in weight. Wells and Sutton (1915) found an average of 9,646,000 corpuscles per cubic millimeter of blood in the cat, but made determinations on only 2 animals. The average normal count for the 16 cats reported here was 8,922,500 red corpuscles per cubic millimeter. Powers, Bowie, and Howard (1930) made red-corpuscle counts on 25 normal, male dogs and found an average of 7,002,000 corpuscles per cubic millimeter, with variations of from 5,070,000 to 8,610,000 per cubic millimeter, which variations are in accord with variations found in the 16 cats herein reported.

Since Barcroft and Barcroft (1923) established that the spleen functions as a reservoir for the red corpuscles, many workers have shown that various physiological states may result in a contraction of the spleen and an increase in the number of red corpuscles present in the circulating blood. More recently, several investigators have reported contraction of the spleen under conditions of excitement or emotion. In a study on man, Benhamou, Marchioni, and Nouchy (1929) found an increase of 9 per cent in the number of red corpuscles present in the circulating blood when an unexpected explosion was used as the exciting agent. Barcroft (1930), after a careful study of the effect of emotion on the spleen of the dog, reported a reduction of 50 per cent in the size of the exteriorized spleen of a dog when it heard a cat mew in an adjacent room. It is difficult to make a fair comparison with animals which do not have the same ratios between spleen weight and body weight. The spleen of the cat represents

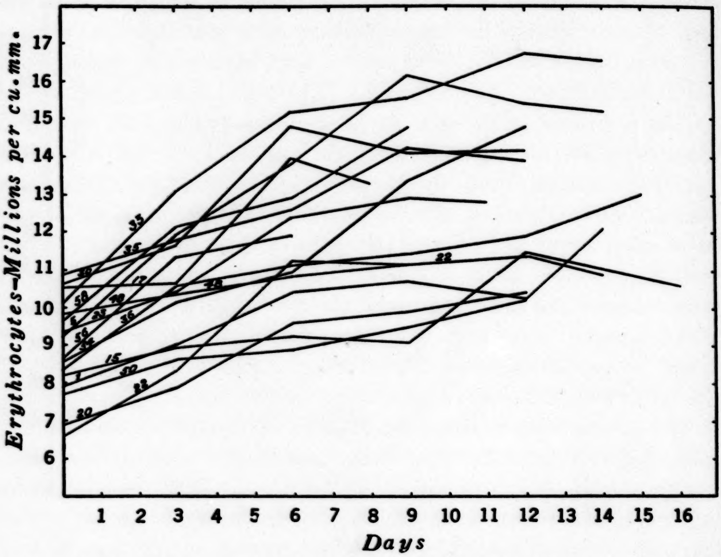

FIG. 2.—Blood concentration from dehydration. The first points on the left indicate the number of erythrocytes prior to dehydration. The number of each experiment is shown by figures on the curves; the duration of dehydration is given in days.

0.05 per cent of body weight, while that of the dog and man represent 0.25 per cent of body weight (Krumbhaar, 1926). Field (1930) found a decided increase in the platelet count of cats when excited from being tied to a board for 3 minutes. In the cat, Cruickshank (1926) found that the blood expelled from the spleen may have 20–40 per cent greater content of hemoglobin than the blood in the general circulation, and that the amount which could be added to the circulation was 2.6–5.6 per cent of the total blood volume. From these figures it may be estimated that the maximum increase in the hemoglobin of the circulating blood following splenic contraction would be less than 2.5 per cent. The cats used in these experiments were not tied when blood samples were drawn, but in many cases the animals were held with considerable force and undoubtedly did experience considerable emotional excitement. From the literature available on this subject it seems probable that an error of 5 per cent may have been introduced into the blood counts through splenic contraction. In view of the high concentrations encountered, this would not materially affect the results. Table IV shows definite

TABLE IV

CHANGES IN BLOOD CONCENTRATION IN RELATION TO DURATION OF DEHYDRATION, RATIO OF WATER INTAKE TO OUTPUT, AND NEGATIVE WATER BALANCE IN PERCENTAGE OF ORIGINAL BODY WEIGHT

Cat. No.	Original Weight (Grams)	Duration of Dehydration (Days)	Ratio of Water Intake to Output	Negative Water Balance in Percentage of Original Weight	Blood Concentration on Last Day in Percentage Increase
1	2,837.0	13.5	1:2.30	28.4	52.58
5	3,080.5	14.0	1:2.90	30.9	65.36
15	1,225.0	16.0	1:1.40	25.1	28.03
17	2,181.0	7.5	1:3.09	22.7	26.42
20	1,943.5	11.5	1:3.47	32.9	50.86
22	3,294.0	13.5	1:3.46	26.3	63.48
23	1,393.5	11.0	1:3.31	33.9	38.67
30	2,737.5	12.0	1:2.00	20.9	34.12
33	3,298.5	14.0	1:2.65	24.2	63.73
35	2,396.0	12.0	1:2.83	31.6	28.49
36	2,730.5	15.0	1:3.33	28.3	56.73
48	2,323.0	9.0	1:10.1	16.6	14.28
50	1,949.5	12.0	1:3.51	29.0	33.93
54	2,266.0	12.0	1:4.66	30.1	72.13
56	1,791.5	6.0	1:18.1	20.2	37.50
58	2,331.5	9.0	1:9.39	19.3	53.31

relations between blood concentration, water intake and output ratios, and negative water balance in percentage of body weight.

The percentage of increase in blood concentration on the last day of the experiment is, in half of the experiments, not the greatest concentration attained. There was often a reduction in concentration near the end of the experiment, especially in the longer experiments. This reduction may have been the result of a failure on the part of the body to replace corpuscles, due to nutritional disturbances. Marriott (1923) reported a decrease in the concentration of hemoglobin and of serum protein after a number of days of severe anhydremia. In a study of the effects of reduced blood volume on tissue maintenance, Rous and Gilding (1929) reported that, after hemorrhage in cats and rabbits, the red bone marrow was well served with blood although situated in limbs almost deprived of a circulation. To some extent starvation may have affected the replacement of red blood corpuscles, although Robertson (1913) reported that in the fasting dog and rat the percentage of hemoglobin and the number of blood corpuscles were not appreciably affected.

White blood corpuscle counts were made throughout the experiments; but, other than a general increase in number with dehydration, there were no significant changes.

RESPIRATORY EXCHANGE DURING DEHYDRATION

The literature relative to the effect of dehydration on gaseous metabolism is very limited. Straub (1899) reported no change in carbon-dioxide production of one dog deprived of water and fed air-dried meat powder for 3 days. Warburg (1909) and Loeb and Wasteneys (1913) reported an increased rate of oxidation in unfertilized sea-urchin eggs when placed in hypertonic solutions. Caldwell (1925) reported increased carbon-dioxide production in several species of animals, including the frog, after dehydration in dry air. Iizuka (1926) desiccated frogs in several ways and at different temperatures and found an increase of more than 55 per cent in carbon-dioxide production following desiccation over sulphuric acid for 24 hours. The increase in oxygen consumption was less, which resulted in an increased respiratory quotient.

When these experiments were undertaken, the question of dehydration in relation to maintenance of body temperature was in a highly unsatisfactory state. Therefore, no attempt was made to measure the respiratory exchanges until seven of these experiments and several preliminary ones indicated that, under the conditions of the experiment, dehydration did not affect body temperature. Air samples were drawn usually 12 hours after feeding, but in some cases less time had elapsed. The samples probably did not always represent post-absorption metabolism, but the increase in metabolism due to digestion must have been small. Sonderstrom, Barr, and DuBois (1918) showed that a small standard breakfast increased the basal metabolism of three men only 7 per cent for the first hour and not at all after 6 hours. Since it is impossible to estimate the degree of relaxation under such circumstances, no claim is made that the metabolism of the experimental animals was at a basal rate; but the standard procedure followed justifies a comparison of control and experimental results. The carbon-dioxide production and oxygen-consumption data were reduced to 0°C., 760 mm. Hg., dry, with corrections for brass scale barometer.

Loewy (1911) found the oxygen consumption of normal cats to be 1.005 gm. per kilogram hour and the carbon-dioxide production to be 1.064 gm. per kilogram hour. The averages for the cats in these experiments are: oxygen consumption 0.892 gm. and carbon-dioxide production 0.899 gm. per kilogram hour. The normal averages are lower than Loewy's figures, but higher than those of Aub, Foreman, and Bright (1922), who used cats in a study of the effect of adrenalectomy on metabolism. These workers made a large number of determinations on 9 normal cats and selected the lowest results as control rates. Their cats also exceeded the average weight of the cats in this report by 34 per cent, which, according to the Law of Surface Area, would result in lower rates when calculated on the basis of body weight. Heat production in calories per square meter of surface were not calculated in these experiments, because the constant used in such calculations was determined on normal cats and because, after a few days of desiccation, the normal weight-surface relations would be in error.

The greatest increases in respiratory exchange were noted between the second and the seventh day of dehydration (Figs. 3 and 4).

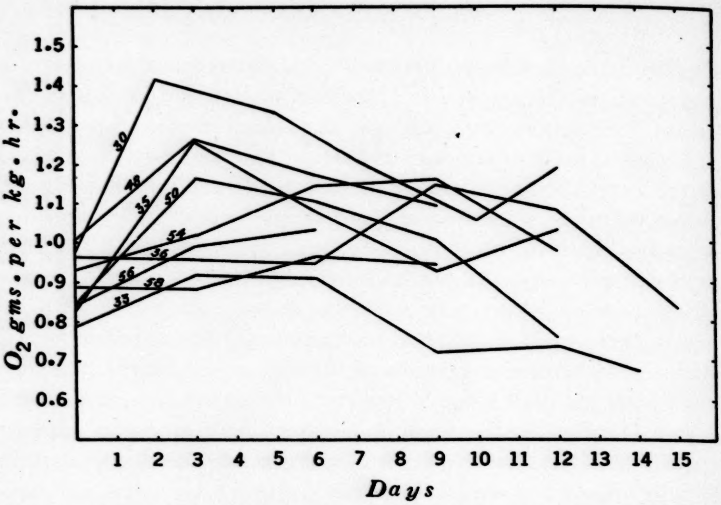

FIG. 3.—The normal oxygen consumption of the cats and the increase or decrease during dehydration. The number of each experiment is shown by figures on the curves; the duration of dehydration is given in days.

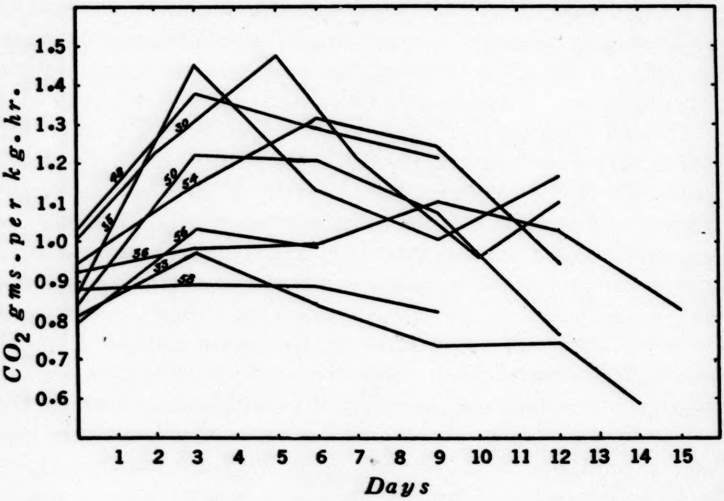

FIG. 4.—The normal carbon-dioxide production of the cats and the increase or decrease during dehydration. Figures on the curves indicate the number of the experiment; the duration of dehydration is given in days.

After the seventh day, there was a gradual decrease, and in experiments extending longer than 9 days an average rate considerably below normal. The greatest increases in oxygen consumption averaged 28.8 per cent. The average increase for all of the experiments and for the entire period of dehydration was 14.9 per cent. The increase in carbon dioxide production was greater than in oxygen consumption. The greatest increases in carbon-dioxide production averaged 33.9 per cent, and the average increase for the duration of dehydration was 18.3 per cent. In the frog (Caldwell, 1925, and Iizuka, 1926), the increase in carbon dioxide was much greater, but the frog gives up its water very quickly when exposed to circulating dry air. There is some evidence that an increase in respiratory exchange occurs before the animal has lost sufficient water to dehydrate the tissues to an appreciable extent. This suggests the possibility that respiratory metabolism is stimulated by the increased rate of evaporation from the mucous membranes in dry air.

Loewy (1911) found the respiratory quotient of normal cats to be 0.77; and Aub, Foreman, and Bright (1922) reported an average respiratory quotient of about 0.78, with variations from 0.73 to 0.96. The normal respiratory quotient of these cats varied from 0.70 to 0.79, and averaged 0.74. Dehydration had no marked effect on the respiratory quotient. With one exception, the respiratory quotient increased slightly for the first 3 days of dehydration (Fig. 5). This may have been the result of increased blood flow to the mucous membranes to supply moisture lost from contact with dry air, which would result in greater ventilation of the blood. The decrease in respiratory quotient characteristic of a fasting condition (Benedict, 1915) did not appear in these experiments.

METABOLIC-WATER PRODUCTION

Water formed in the metabolic processes of the body may be considered, according to its source, as intramolecular water, water of oxidation, and water of syntheses. The term "metabolic water" generally refers to the total water produced from all three sources. Intramolecular water may be liberated from oxidized organic material supplied as food or from organic material synthesized in the body. In the second event the oxygen which united with hydrogen to form water would be an addition to the body weight as a result of respiration. In the production of water of oxidation all of the oxygen which united with hydrogen to form water would result in an increase in body weight.

Water production as a result of metabolism has been recognized since the early work in chemical physiology; and estimates for man, based on computations of the amount of hydrogen oxidized, vary from 150 to 400 gm. per day. Babcock (1912) pointed out that the same carbon nucleus may function several times in hydrolytic and dehydrating reactions, and suggested that metabolic water has a function which cannot be served by imbibed water. Since metabolic water is produced within the cell, it reduces the concentration of the cell contents and thereby disturbs the osmotic equilibrium between the fluids without and within the cell, with the result that nutrients move to the region of lower concentration; while imbibed water causes reduction of concentration on the outside of the cell which aids elimination from the cell but hinders the movement of nutrients into the cell. Babcock has also shown that metabolic-water production is of particular importance to animals living on air-dried food, such as the clothes moth, pea weevil, and other insects which do not have access to free water.

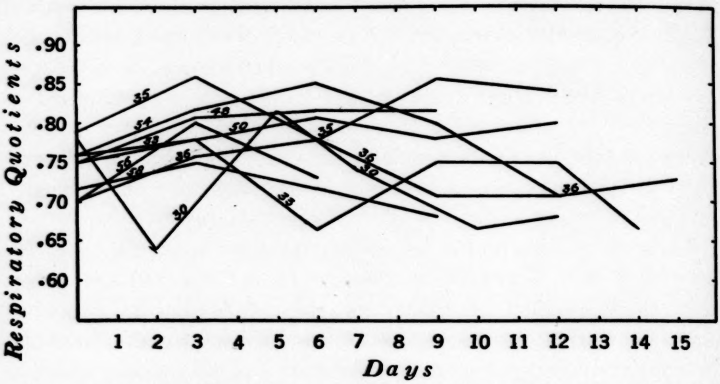

FIG. 5.—The normal respiratory quotients of the cats and the effect of dehydration on the respiratory quotient. Figures on the curves indicate the number of the experiment; the period of dehydration is given in days.

An attempt has been made in these experiments to determine the quantity of metabolic water produced by cats while undergoing a slow but severe dehydration. Protein yields less metabolic water than other foodstuffs, i.e., 41 per cent as compared to 55 per cent in carbohydrates, and 110 per cent in fat metabolism. For this reason, a diet high in protein content, made up only of lean meat from which all visible fat had been removed, was given the animals for several weeks prior to and throughout the experiment. This, obviously, does not mean that fat and glucose in addition to that present at the beginning of the experiment is not oxidized; but fats and glucose formed by syntheses, when oxidized, give up the intramolecular water acquired in the process of synthesis as water of metabolism.

The metabolic water produced in these experiments was first calculated from the total intake and output of water, assuming that the total loss in weight of the cat was water and making a correction of these figures for carbon loss in carbon-dioxide excretion and for the additions to the weight of the cat in the form of dry weight of the food. It was then found that precisely the same results were obtained from calculations based on the total intake and output of the cat in relation to its initial and final weights. This gave the addition to the weight of the cat in oxygen absorbed but not excreted as carbon dioxide. Since the hydrogen utilized in the production of metabolic water was already present, the actual weight of the water so formed would be 1.1118 times that of the oxygen utilized. As the latter method is less complicated and all experiments have been calculated by both methods with identical results, only the latter method will be presented in detail. All totals for the determinations as given below were taken from the data on experiment No. 36. On account of the large volume of data, it is only possible to present results in brief form.

INTAKE

250.59 gm. dry weight of food
331.91 gm. water in food
582.50 gm. total ingesta

OUTPUT

471.00 gm. urine
616.00 gm. water collected in desiccator

19.53 gm. water in feces
18.67 gm. solids in feces
234.48 gm. carbon excreted as carbon dioxide

1359.68 gm. total egesta

2730.50 gm. original weight of cat
582.50 gm. total ingesta

3313.00 gm. original weight of cat+total ingesta
1359.68 gm. total egesta

1953.32 gm. weight of cat if no water had been formed

2140.50 gm. actual final weight of cat
1953.32 gm. final weight if no water had been formed

187.18 gm. addition to weight of cat from oxygen utilized in metabolic-water production
187.18×1.1118=208.10 gm. metabolic water produced

An estimate of the calorie production, based on an average caloric value of a liter of oxygen as calculated from the tables of Benedict and Fox (1925), showed that the total calorie production in each experiment was at least 20 per cent greater, with the exception of experiment No. 15, than the possible calorie production from the food consumed by the animal. Since the animals, therefore, must have metabolized some of their own tissues, it seems reasonable to assume that none of the oxygen retained under the conditions of the experiment was utilized in tissue production. Solids in the urine do not affect the total of metabolic water produced, when calculated on the basis of entire intake and output. The metabolic water in grams per kilogram day was calculated from the average weight of the cat, determined from the morning and evening weights.

Table V shows the metabolic-water production for each experiment in relation to loss of weight, water intake-output ratios, and duration of the experiment for both the entire period and for each half. These results give only the minimum metabolic-water production and do not take into consideration intramolecular water from nutrients not synthesized in the animal. Some water was undoubtedly lost in collection of urine from the pans; and all water from respiration was lost while the animals were out of the respiratory

TABLE V

METABOLIC-WATER PRODUCTION IN RELATION TO BODY WEIGHT, RATIO OF WATER INTAKE AND OUTPUT, AND DURATION OF DEHYDRATION

CAT NO.	ENTIRE PERIOD OF DEHYDRATION					FIRST AND LAST HALF DEHYDRATION PERIODS		
	Initial Weight (Grams)	Loss in Weight (Grams)	Duration of Dehydration (Days)	Ratio of Water Intake to Output	Metabolic Water Produced (Grams per Kilogram Day)	Duration of Dehydration (Days)	Ratio of Water Intake to Output	Metabolic Water Produced (Grams per Kilogram Day)
1....	2,837.0	549.5	13.5	1:2.30	4.67	6.5 7.0	1:2.27 1:2.34	6.32 3.04
5....	3,080.5	682.0	14.0	1:2.90	4.93	7.0 7.0	1:3.07 1:2.65	4.15 5.82
15....	1,225.0	3.5	16.0	1:1.40	4.65	8.0 8.0	1:1.17 1:1.88	6.04 3.25
17....	2,181.0	390.0	7.5	1:3.09	6.73	3.5 4.0	1:2.18 1:6.60	5.75 7.69
20....	1,943.5	463.5	11.5	1:3.47	4.52	5.5 6.0	1:4.80 1:2.76	5.18 3.86
22....	3,294.0	666.0	13.5	1:3.46	5.66	6.5 7.0	1:3.50 1:3.39	4.81 6.57
23....	1,393.5	340.5	11.0	1:3.31	6.11	5.5 5.5	1:3.65 1:2.92	5.30 7.03
30....	2,737.5	353.5	12.0	1:2.00	6.33	6.0 6.0	1:1.60 1:2.94	6.36 6.29
33....	3,298.5	537.0	14.0	1:2.65	4.01	7.0 7.0	1:2.47 1:3.07	4.24 3.71
35....	2,396.0	514.5	12.0	1:2.83	6.31	6.0 6.0	1:2.45 1:3.96	7.25 5.23
36....	2,730.5	590.0	15.0	1:3.33	5.65	7.5 7.5	1:2.83 1:4.46	6.16 5.08
48....	2,323.0	368.5	9.0	1:10.1	8.07	4.5 4.5	1:7.02 1:23.0	7.90 8.25
50....	1,949.5	429.5	12.0	1:3.51	5.31	6.0 6.0	1:3.72 1:3.29	4.60 6.11
54....	2,266.0	579.0	12.0	1:4.66	7.11	6.0 6.0	1:3.30 1:15.9	6.45 7.85
56....	1,791.5	343.0	6.0	1:18.1	8.46	3.0 3.0	1:12.4 1:84.4	8.73 8.16
58....	2,331.5	418.0	9.0	1:9.39	6.60	4.5 4.5	1:5.73 1:174.0	5.66 7.63

chamber, approximately 2 hours each day. These losses would have increased the total output from the animal and thereby increased the calculated metabolic water produced to the same extent. The carbon loss would have been more accurate had it been possible to make continuous carbon-dioxide-production determinations. For the experiments in which gaseous exchange was not determined, the carbon loss was estimated on the basis of the average per kilogram hour for the experiments in which carbon-dioxide production was determined.

No literature has been found on metabolic-water production in animals lower than man; therefore it is difficult to make comparisons. Rogers (1928) estimated, on the basis of diet, a metabolic-water production in man of 300–400 gm. per 24 hours. For a 75-kg. man this would average from 4 to 5.3 gm. per kilogram day. The average for the 16 cats in these experiments was 5.94 gm. per kilogram day. Benedict and Carpenter (1910) made a large number of determinations on the metabolism of healthy man during rest and found an oxygen consumption of approximately 0.367 gm. per kilogram hour. The average normal oxygen consumption of the cats in these experiments was 0.892 gm. per kilogram hour, or a rate 2.4 times as great as that in man. Since metabolic-water production is dependent, in part, upon rate of oxidation, if the estimates on metabolic-water production in man are correct, the cats should have produced from 9.6 to 12.7 gm. of metabolic water per kilogram day, or almost twice the average minimum quantity measured in these experiments. This would have been possible had a mixed diet been used and the intramolecular water included.

DISCUSSION

Because of the importance of water in all of the vital processes of animals, the physiological responses to a disturbance of water balance are undoubtedly numerous and of far-reaching consequences. Experiments of various kinds have shown that water is more vital than food but less vital than oxygen. Many physiological effects from a derangement of the equilibrium of water metabolism could probably be referred to histological changes in certain organs and tissues of the body if sufficient data were available for correlation.

Garofeanu and Derevici (1924) studied the histological changes in the tissues of dogs deprived of water from 4 to 9 days, and reported lesions in the tissues of many organs even from the first day. The lungs, the liver, and the thyroid suffered to the greatest extent from water deficiency; but lesions appeared in the intestinal mucosa, adrenals, kidneys, and all other glandular tissues studied. The latter is in accord with the results of Kudo (1921*b*), who found the greatest proportional losses in weight in the glandular structures of the body when rats were deprived of water. Since many physiological reactions are subject to modifications through hormone action, it would be expected that changes would occur with stimulation or depression of the activity of the endocrine glands from dehydration. Sufficient knowledge is not available on the effects of water losses to predict either hyperactivity or hypoactivity following lesions from water deficiency. Since the thyroids control, to a considerable extent, the respiratory metabolism of animals, it is not inconceivable that the increase in gaseous metabolism found in dehydrated cats may have resulted from stimulation of the thyroids due to lesions produced by water losses; however, there is no experimental evidence that this was even a contributory factor. Hammett (1927) ascribed the anhydremia which accompanies thyroid and parathyroid deficiency to lowered nutritional level rather than to any specific relation of the glands to water balance. There is considerable evidence that the posterior lobe of the hypophysis functions in the maintenance of normal water balance, but the mechanism is unknown. Priestley (1921) determined that the diuresis induced by water-drinking could be delayed for 4–6 hours by injections of pituitrin. The diuresis is then accompanied by an increased excretion of chlorides. Weir, Larson, and Roundtree (1922) have shown from experiments on dogs that the antidiuretic effect of pituitary extract is independent of the nerve supply of the kidney. Polydypsia and polyuria are recognized symptoms of diabetes *insipidus* and are related to deranged water metabolism. Roundtree has reported urinary outputs from diabetes *insipidus* cases in excess of 14 liter per day, which could be temporarily reduced by extracts of the posterior lobe of the hypophysis. Adolph and Ericson (1927) showed that the kidney of man is insensitive to an excess of water in the blood when pituitrin is administered. Downs and Geiling (1929) showed that mice which received alkaline extracts of the anterior lobe of the pituitary gained weight more rapidly than controls but that their tissues contained from 6 to 8 per cent more water than the tissues of controls. Some work indicates that the hypophysis is only of minor importance in water-balance equilibrium. Camus and Roussy (1920) removed the hypophysis from dogs and reported only a transitory polyuria following the operation. More than a month later, when the animals had recovered from the first polyuria, a second polyuria was produced by puncture of the tuber cinereum. Bailey and Bremer (1921), Rogers (1924*b*), Kayser (1929), and others have produced polyuria and blood concentration by lesions of various parts of the midbrain without injury to the hypophysis; however, this does not eliminate the possibility that injury to nerve tracts connecting with the hypophysis may have been the immediate cause. Molitor and Pick (1925) and Hoffman and Wertheimer (1927) have suggested a central nervous control of water balance in the midbrain, but conclusive proof is not presented. Liu and Krüger (1927) reported a polyuria and hydremia after midbrain puncture and concluded that certain midbrain regions not only controlled kidney function but influenced vascular permeability and tonus.

Kudo (1921*a*) reported no loss in weight of the brain and spinal cord in rats deprived of water; but in a study of the effects of withdrawal of water on the salivary conditioned reflex induced by morphine, Crisler (1928) found that conditioned secretion of saliva in the dog was practically abolished after 3 to 5 days of dehydration. This worker reported that the peripheral mechanism was little affected, and concluded that dehydration resulted in a depression of certain central processes. The dehydrated cats reported here showed a marked change in behavior which suggests a depression of sensory centers; this fact is difficult to understand, when accompanied by an increase in respiratory exchange, unless dehydration of peripheral nerves increased muscular tonus.

In the dehydration of body tissues the movement of water from the intercellular and intracellular spaces to the blood vessels must take place against the hydrostatic pressure within the capillaries. The colloid osmotic pressure of the plasma proteins must play an important rôle in this absorption process. Landis (1928) measured the pressure in the arteriolar end and venous end of the capillaries of the mesentery of rats and guinea pigs and found an average arteriolar pressure of from 30 to 40 cm. of water and an average venous pressure of 11.5 cm. of water. The osmotic pressure of the plasma proteins was 22–27.5 cm. of water. Under these circumstances, filtration of water would take place at the arteriolar end and absorption at the venous end. With a reduced volume of blood, as in dehydration, the filtration pressure would decrease while the increased concentration of the blood would result in a higher osmotic pressure of the colloids and a greater movement of water into the blood stream. Inanition from starvation would accentuate the concentration of blood through a decrease in plasma proteins but would tend to lower the dehydration rate of the tissues. For this reason, the results of experiments on starved animals deprived of water are hardly comparable to experiments on animals deprived of water with food *ad libitum*.

Clinical evidence of disturbance of water balance associated with the symptom fever has been noted so widely that a review of the literature at this time would be superfluous. The effect of disturbance of water balance on body temperature has received much less attention, although pediatricians have long recognized the clinical significance of acute water loss in inanition fevers and retention toxicosis in infants. Schiff (1929) points out that uncomplicated pictures of water deficiency are rarely seen on the sick bed. By controlling the diet, Schiff and his co-workers have reduced the water intake of a large number of children to about 40–50 per cent of the normal needs, and report an increase in body temperature in half of the cases, with wide individual differences in symptom complexes. If reduction of free water by an increased capacity for fixation by the colloids is responsible for the symptom fever, as proposed by Balcar, Sansum, and Woodyatt (1919), the animals in these experiments should have demonstrated a hyperthermia at some stage of dehydration, since, while not so rapid, the reduction of free water was greater. Andrews (1926) concluded from his experiments on dogs that a low-grade fever is always present in dehydrated animals. These results fail to confirm such a conclusion.

While these experiments have been an attempt to study some phases of water metabolism in a laboratory animal under severe conditions, the results may have some significance in the water relations of desert animals, which must encounter even more severe conditions in their normal environments.

The facts that the only cat of this series which was fed uncooked beef consistently gained in weight with a reduced water intake and that dehydrated cats which had refused cooked meat for several days would eat raw meat may indicate that carnivorous animals in an arid region are quite independent of free water for long periods. However, the herbivorous animals of desert and semidesert regions, some of which feed largely on air-dried seeds and grasses and will not drink when water is available (Bailey, 1923), have no comparable source of water. Metabolic water furnishes a constant supply, which may be augmented by increased respiratory metabolism when obligatory water has been reduced to a minimum; but the amount of metabolic water produced is directly dependent upon the free hydrogen available from oxidation of food or tissues of its own body, and therefore the quantity is necessarily limited.

No physicochemical mechanism for water conservation such as that which occurs in birds and scaly reptiles in the excretion of uric acid crystals has been described for mammals. Whether the maintenance of normal water balance is regulated through the nervous system, through hormone action, through physicochemical changes, or a combination of these cannot at present be predicted; but the adaptation of certain mammals to the reduced water intake of desert conditions must involve important physiological changes in organs which have to do with nutrition and elimination; the investigation of these not only should add materially to the scanty knowledge of the physiology of desert animals but should contribute to the general physiology of water metabolism, which in turn is fundamental to a proper understanding and solution of one of the most important problems of the ecology of desert animals.

SUMMARY

1. Adult cats have been dehydrated by water deprivation in dry air, without acute starvation, to such an extent that the

negative water balance averaged 26.8 per cent of the original body weight.

2. The average increase in blood concentration which resulted from dehydration was in excess of 45 per cent of the original blood volume. An increase of 75 per cent was recorded without fatal results.

3. The maintenance of normal body temperature was not affected by dehydration from water deprivation. There was no indication that a reduction of free water results in an increase in body temperature.

4. Metabolic-water production was not noticeably increased with increasing severity of dehydration. There was some evidence that cats could live for long periods on a diet of flesh and blood, such a diet as might be obtained from a recently killed animal.

5. Dehydration increased the total respiratory exchange of the cats without marked effect on the respiratory quotient. There was a decided decrease in respiratory exchange before the vital limit of dehydration was reached.

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